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CONTRACTING ORGANIZATION: Oregon Health Sciences University
Portland, Oregon 97201

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1 March 1992   Final Proceedings

Bone symposium '91

Alan E. Seyfer, M.D.

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U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

Symposium held 17-20 July 1991

Conference, Bone

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BONE SYMPOSIUM '91
AGENDA

17 JULY 91 (WEDNESDAY)

8:05 - 8:10
Welcome
Jeffrey O. Hollinger, DDS, PhD, COL, US Army
Chief, Physiology Branch
US Army Institute of Dental Research
Walter Reed Army Medical Center
Co-Chairman

Opening Remarks
Donald D. Trunkey, MD
Professor and Chairman
Department of Surgery
Oregon Health Sciences University

CLINICAL SESSION I: 8:00 - 12:00, Marriott Hotel
Session Leader
Alan E. Seyfer, MD
Professor of Survery and Chairman
Division of Plastic and Reconstructive Surgery
Oregon Health Sciences University
Co-Chairman

8:10 - 9:00
Osteomyelitis: Etiology and Local Considerations
Jon Mader, MD
Professor of Medicine
The Marine Biomedical Institute
Division of Infectious Diseases
University of Texas Medical Branch

9:00 - 9:50
Adult Osteomyelitis Treatment Protocol (1991)
George Cierny, MD
Associate Professor of Surgery
Department of Orthopedics
Emory University of Medicine
Crawford W. Long Hospital of Emory University

9:50 - 10:15
Coffee Break

10:15 - 11:10
The Current Status of Bone and Cartilage Allografting
Henry Mankin, MD
Chief, Orthopedic Surgery
Orthopaedic Oncology Unit and Bone Bank
Massachusetts General and Children's Hospitals
Harvard Medical School

11:10 - 12:00
Vascularized Bone Grafts in Clinical Surgery
James R. Urbaniak, MD
Division of Orthopaedic Surgery
Duke University Medical Center

12:00 - 1:30
Lunch

CLINICAL SESSION II: 1:30 - 5:30, Marriott Hotel
Session Leader
Alan E. Seyfer, MD
Professor of Surgery and Chairman
Division of Plastic and Reconstructive Surgery
Oregon Health Sciences University

1:30 - 2:25
The Ilizarov Technique: A Method to Regenerate Bone and Soft Tissue
Dror Paley, MD
Assistant Professor of Orthopedic Surgery
Division of Orthopedic Surgery
University of Maryland Hospital

2:25 - 3:20
Bone Graft Systems in Oral and Maxillofacial Surgery
Phil Boyne, MS, DDS, DSc (Hon.)
Chief, Oral and Maxillofacial Surgery
Loma Linda University Medical Center

3:20 - 3:40
Coffee Break

3:40 - 4:35
Bone Grafts and Reconstruction of Tumors of the Skull
Ian Jackson, MD
Institute of Craniofacial and Reconstructive Surgery

4:35 - 5:30
Toward the 21st Century: CT Based Facial Fracture Treatment
Paul Manson, MD
Professor of Plastic Surgery
Maryland Institute for Emergency Medical Services Systems
The Johns Hopkins Hospital

6:00 - 7:00
Reception - Marriott Hotel

18 JULY 91 (THURSDAY)

Research Session I: 8:00 - 12:00, Marriott Hotel
Session Leader
Jeffrey O. Hollinger, DDS, PhD, COL, US Army
Chief, Physiology Branch
USArmy Institute of Dental Research
Walter Reed Army Medical Center

8:00 - 8:55
Histomorphometry of Surgical, Biomechanical, and Gravitational Effects at the
Macroscopic and Microscopic Levels
Eugene Roberts, DDS, PhD
Department of Orthodontics
Indiana School of Dentistry

8:55 - 9:50
Fundamentals of Histometry for Bone Graft Substitutes
Ralph Holmes, MD
Associate Professor
Division of Plastic Surgery
University of California - San Diego

9:50 - 10:15
Coffee Break

10:15 - 11:10
Growth Factors: An Opportunity to Manipulate Healing at the Cellular and
Molecular Level
Michael Joyce, MD
Department of Orthopedic Surgery
Washington University School of Medicine

11:10 -12:00
Correlation Between Gene Expression and Histology Suggests Local Regulation of
Fracture Repair
Mark Bolander, MD
Department of Orthopedic Surgery Laboratory
The Mayo Clinic

12:00 - 1:30
Lunch

RESEARCH SESSION II: 1:30 - 5:30, Marriott Hotel
Session Leader
Leslie J. Marden, PhD, CPT, US Army
Biochemistry - Physiology Branch
US Army Institute of Dental Research
Walter Reed Army Medical Center

1:30 - 2:20
The TGF -β Family and Bone Remodeling
Mike Centrella, PhD
Associate Professor
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Endocrine Division
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2:20 - 3:15
Tissue Repair and Growth Factors
Glenn Pierce, MD, PhD
Department of Experimental Pathology
Amgen

3:15 - 3:35
Coffee Break

3:35 - 4:35
Current Status of Bone Morphogenetic Proteins and Osteogenin
A. H. Reddi, PhD
Chief, Bone Cell Biology Section
National Institute of Dental Research
National Institutes of Health

4:35 - 5:30
Clinical Applications of Molecular Engineering: Bone and Cartilage Repair
Tom Einhorn, MD
Associate Professor of Orthopaedics
Director of Orthopaedic Research
Mt. Sinai Medical Center

19 JULY 91 (FRIDAY)

CLINICAL SESSION III: 8:00 - 12:00, Marriott Hotel
Session Leader
Rodney Beals, MD
Professor/Head, Orthopedics and Rehabilitation
Oregon Health Sciences University

8:00 - 8:55
Laboratory and Clinical Experience with a Synthetic Bone Graft Substitute
Charles Cornell, MD
The Research Division
The Hospital for Special Surgery
Cornell University Medical College

8:55 - 9:50
Biologic and Immunologic Aspects of Bone Grafts
Gary Friedlaender, MD
Professor and Chairman
Department of Orthopedics and Rehabilitation
Yale University School of Medicine

9:50 - 10:15
Coffee Break

10:15 - 11:05
AIDS and Bone Grafts: Recommendations
William W. Tomford, MD
Department of Orthopaedic Surgery
Massachusetts General Hospital

11:05 - 12:00
Bone Grafts and Alloplastic Materials in Neurosurgery
Don Prolo, MD
Clinical Associate Professor of Surgery
Stanford University School of Medicine

12:00 - 1:30
Lunch

DELIVERY SYSTEMS FOR BONE GROWTH AND INDUCTIVE FACTORS
1:30 - 5:30, Marriott Hotel
Session Leader
Jeffrey O. Hollinger, DDS, PhD, COL, US Army
Chief, Physiology Branch
US Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, DC

1:30 - 2:25
Ceramic Materials and Bone Repair
Myron Spector, PhD
Director and Lecturer
Orthopedic Research Department
Brigham and Women's Hospital

2:25 - 3:20
Polymers for Bone Regeneration: Requirements and Applications
Carla Desilets, PhD, CPT, US Army
Analytical Chemistry, Physiology Branch
US Army Institute of Dental Research
Walter Reed Army Medical Center

3:20 - 3:40
Coffee Break

TABLE OF CONTENTS

VII. INVITED PAPER

3:40 - 4:35
Polymers
Robert Langer, D.Sc.
Department of Chemical Engineering
Harvard-MIT Division of Health Sciences and Tehchnology
Whitake College of Health Sciences
Massachusetts Institute of Technology

4:35 - 5:30
Collagen and Bone Repair
Edward J. Miller, PhD
Department of Biochemistry
University of Alabama at Birmingham

6:30 - 7:30, SOCIAL   HOUR, Marriott Hotel

7:30 - 10:00, BANQUET, Marriott Hotel

Keynote Address:
Historical Perspective: Bone Induction
Marshal Urist, MD
Professor, Department of Surgery
Bone Research Laboratory
University of California, Los Angeles

20 JULY 91 (SATURDAY)

CONTROVERSIES I: BONE REGENERATION FACTORS
                    DELIVERY SYSTEMS
                    BONE "BEHAVIOR"
8:00 - 10:00 Oregon Health Sciences University

Marshall Urist, MD - Moderator
Professor, Department of Surgery
Bone Research Laboratory
University of California, Los Angeles

Panelists
David Baylink, MD
Mike Centrella, PhD
Harold Frost, MD
Jack Lemons, PhD
Myron Spector, PhD
A. H. Reddi, PhD
Bob Langer, DSc

CONTROVERSIES II: TYPES OF GRAFTS
REQUIREMENTS
INFECTIONS - TREATMENTS
10:00 - 12:00 Oregon Health Sciences University

Gil Triplett, DDS, PhD - Moderator
Chairman and Professor, Department of Oral and Maxillofacial Surgery

Panelists
Gary Bos, MD
Phil Boyne, MS, DDS
Hans Burchardt, PhD
George Cierny, MD
Tom Einhorn, MD
Mutaz Habal, MD
Robert Marx, DDS
William Tomford, MD

OSTEOMYELITIS: ETIOLOGY AND LOCAL CONSIDERATIONS

Jon T. Mader, M. D.[1,2]

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Jason Calhoun, M. D.[3]

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INTRODUCTION

The term osteomyelitis refers to an inflammation of the marrow (myelitis) of the bone(osteo). However, the diagnosis is generally made in reference to an infection involving the cortical and/or medullary portions of a bone. Based on etiologic and clinical considerations, bone infections have traditionally been classified as either hematogenous osteomyelitis or osteomyelitis secondary to contiguous focus of infection. Contiguous focus osteomyelitis has been further subdivided into osteomyelitis in patients who have relatively normal vascularity and patients with generalized vascular insufficiency.

Osteomyelitis may be acute or chronic. The acute disease is characterized by a suppurative infection accompanied by edema, vascular congestion, and small vessel thrombosis. The vascular supply to the bone is compromised as the infection extends into the surrounding soft tissue. Large areas of dead bone (sequestra) may be formed when both the medullary and periosteal blood supplies are compromised. Viable colonies of bacteria may be harbored within the necrotic and ischemic tissues even after an intense host response, surgery, and/or therapeutic antibiotics. Once the antibiotics are discontinued or the host response declines, the organisms may again proliferate and lead to a recurrence of the infection. The hallmarks of chronic osteomyelitis are a nidus of infected dead bone or scar tissue, an ischemic soft tissue envelope, and a refractory clinical course.

The authors will discuss the etiology of the standard classification of osteomyelitis. An alternative staging system of osteomyelitis based on the four major factors influencing the treatment and prognosis will be presented. Finally, local therapy of

osteomyelitis using antibiotic impregnated polymethylmethacrylate (PMMA) and biodegradable beads will be explored.

HEMATOGENOUS OSTEOMYELITIS

Hematogenous osteomyelitis occurs mainly in infants and children. The metaphysis of the long bones (tibia, femur) are most frequently involved. The anatomy in the metaphyseal region seems to explain this clinical localization.[1] Non anastomosing capillary ends of the nutrient artery make sharp loops under the growth plate and enter a system of large venous sinusoids where the blood flow becomes slow and turbulent. The metaphyseal capillaries lack phagocytic lining cells and the sinusoidal veins contain functionally inactive phagocytic cells.[2] These capillary loops are essentially terminal branches of the nutrient artery. Any end capillary obstruction could lead to an area of avascular necrosis. Minor trauma probably predisposes the infant or child to infection by producing a small hematoma, vascular obstruction, and a subsequent bone necrosis which is susceptible to innoculation from a transient bacteremia.[3] The acute infection initially produces a local cellulitis which results in a breakdown of leukocytes, increased bone pressure, decreased pH, and decreased oxygen tension. The cumulative effects of these physiologic changes further compromise the medullary circulation and enhances the spread of infection.

The infection may proceed laterally through the Haversian and Volkmann canal systems, perforate the bony cortex, and separate the periosteum from the surface of the bone. When this occurs in the presence of medullary extension, both the periosteal and endosteal circulations are lost and large segments of dead cortical and cancellous bone are formed. In the infant, the medullary infection may spread to the epiphysis and joint surfaces through capillaries which cross the growth plate. In a child over one year of age, the growth plate is avascular and the infection is confined to the metaphysis and diaphysis. The joint is usually spared unless the metaphysis is intracapsular. Thus, cortical perforation at the proximal radius, humerus, or femur infects the elbow, shoulder, or hip joint, respectively, regardless of the age of the patient.

Since infants and children with hematogenous osteomyelitis usually have normal soft tissue enveloping the infected bone and are capable of a very efficient metabolic response to infection, they have the potential to resorb large sequestra and generate a

significant periosteal response to the infection. This latter feature leads to substai. ' ' formation of bone at the margin of the infection called involucrum. The involucrum affords skeletal continuity and a maintenance of function during the healing phase.

A single pathogenic organism is almost always recovered from the bone in hematogenous osteomyelitis. Polymicrobic hematogenous osteomyelitis is rare.[4] In the infant, *Staphylococcus aureus*, Group B streptococcus, and *Escherichia coli* are the most frequently recovered bone isolates. Whereas, in children over one year of age, *S. aureus*, *Streptococcus pyogenes*, and *Haemophilus influenza* are the most common organisms isolated. However, after age four the incidence of *H. influenza* osteomyelitis declines.

Hematogenous osteomyelitis is also found in the adult population. The infection usually begins in the diaphysis but may spread to involve the entire medullary canal. Extension into the joint may occur since the growth plate has matured and once again shares vessels with the metaphysis. Cortical penetration usually leads to a soft tissue abscess as the periosteum is firmly adherent to the bone. Subperiosteal abscesses and massive cortical devitalization rarely occur. In time, sinus tracts will form connecting the sequestered nidus of infection to the skin via the soft tissue extension(s). In the adult, *S. aureus, S. epidermidis* and aerobic Gram negative organisms account for the majority of the bone or blood isolates.

VERTEBRAL OSTEOMYELITIS

Vertebral osteomyelitis in the adult patient population is usually hematogenous in origin but may be secondary to trauma. There is often a preceding history of urinary tract infection or intravenous drug abuse.[4,5] An early involvement of the anterior inferior edge of the vertebral body suggests spread from the bony entrance of the anterior spinal artery. However, retrograde infection via Batson's plexus of veins is also postulated.[6] The lumbar vertebral bodies are most often involved followed in frequency by the thoracic and cervical vertebrae. Spread to adjacent vertebral bodies may occur rapidly via the rich venous networks in the spine.[7] Posterior extension may lead to epidural and subdural abscesses or even meningitis. Extension anteriorly or laterally may lead to paravertebral, retropharyngeal, mediastinal, subphrenic, retroperitoneal, or psoas abscesses.

The infection is usually monomicrobic when hematogenous in origin. The most common organism isolated is S. aureus. However, aerobic Gram negative rods are

3

found in 30 percent of the cases. *Pseudomonas aeruginosa* and *Serratia marcescens* have a high incidence of isolation among intravenous drug abusers.

CONTIGUOUS FOCUS OSTEOMYELITIS
WITH NO GENERALIZED VASCULAR INSUFFICIENCY

In contiguous focus osteomyelitis, the organism may be directly innoculated into the bone at the time of trauma or may extend from adjacent soft tissue infections. Common predisposing conditions include open fractures, surgical reduction therapy and internal fixation of fractures, chronic soft tissue infections, and radiation therapy. In contrast to hematogenous osteomyelitis, multiple bacterial organisms are usually isolated from the infected bone. The bacteriology is diverse, but *S. aureus* remains the most commonly isolated pathogen. In addition, aerobic Gram negative bacilli and anaerobic organisms are frequently isolated. Bone necrosis, soft tissue damage, and loss of bone stability occur often making this form of osteomyelitis difficult to manage. The long bones are most frequently involved.

CONTIGUOUS FOCUS OSTEOMYELITIS
WITH GENERALIZED VASCULAR INSUFFICIENCY

The small bones of the feet are commonly involved in this category of osteomyelitis. Inadequate tissue perfusion predisposes the patient to the infection by blunting the local inflammatory response. The infection commonly develops following minor trauma to the feet, infected nail beds, cellulitis, or trophic skin ulceration. Multiple bacteria are usually isolated from the infected bone. The most common organisms are *S. aureus, S. epidermidis, Enterococcus sp.,* Gram negative rods, and anaerobes. Even after presumed successful treatment, recurrence or reinfection occur in the majority of the patients.

CHRONIC OSTEOMYELITIS

Both hematogenous and contiguous focus osteomyelitis can advance to a chronic bone infection. No precise criteria distinguish acute from chronic osteomyelitis. Clinically, newly recognized bone infections are considered acute, whereas, a relapse of a treated infection represents a chronic process. However, this simplistic classification is

4

clearly inadequate. As mentioned, the hallmark of chronic osteomyelitis is the simultaneous presence of organisms, necrotic bone, and a compromised soft tissue envelope. The infection will not resolve until the nidus for the recurrent contamination is removed. Persistent drainage and/or sinus tract(s) are common.

Multiple species of bacteria are usually isolated from biopsies of infected granulations from deep within the wound. Chronic hematogenous osteomyelitis is the exception to this statement since a single organism is often recovered from these patients even years of intermittent drainage. The possibility of attenuating the infection is reduced when the integrity of the soft tissue surrounding the infection is poor or the bone itself is unstable secondarily to an infected nonunion or a septic joint.

STAGING OF OSTEOMYELITIS

There are four major factors influencing the treatment and prognosis of osteomyelitis: 1) the degree of necrosis, 2) the condition of the host, 3) the site and extent of involvement, and 4) the disabling effect of the disease itself. These factors must be considered when assessing treatment results and efficacy of treatment alternatives.

The current classification of hematogenous and contiguous focus osteomyelitis with or without generalized vascular insufficiency is inadequate and imprecise. It does not take into consideration the anatomic nature of the disease or host characteristics. It is unhelpful in determining treatment or identifing prognostic factors. An alternate classification has ' een developed By Cierny and Mader[8,9] which includes these factors (Table 1). In their model the infection and host are staged using four anatomic types and three physiologic classes. The paradigm is determined by the status of the disease process regardless of its etiology or regionality. The anatomic types of osteomyelitis are medullary, superficial, localized, and diffuse. Medullary osteomyelitis denotes infection confined to the intramedullary surface of the bone. Hematogenous osteomyelitis and infected intramedullary rods are examples of this anatomic type. Superficial osteomyelitis, a true contiguous focus infection of bone, occurs when an exposed infected necrotic surface of bone lies at the base of a soft tissue wound. Localized osteomyelitis is usually characterized by a full thickness, cortical sequestration which can be removed surgically without compromising bony stability. Whereas, diffuse osteomyelitis is a through–and–through process which usually requires an intercalary

resection of the bone to remedy. Diffuse osteomyelitis includes those infections with a loss of bony stability either before or after debridement surgery.

The patient is classified as an A, B, or C host. An A host represents a patient with normal physiologic, metabolic, and immunologic capabilities. The B host (Table 2) is either systemically compromised, locally compromised, or both. When the morbidity of treatment is worse than that imposed by the disease itself, the patient is given the C host classification. The terms acute and chronic osteomyelitis are not used in this staging system since areas of macronecrosis must be removed regardless of the acuity or chronicity of an uncontrolled infection. The stages are dynamic and interact according to the pathophysiology of the disease; they may be altered by successful therapy, host alteration, or treatment. This staging system provides a framework for describing and developing experimental models of osteomyelitis, planning medical and surgical treatments, and comparing the results of therapy among institutions.

LOCAL CONSIDERATIONS

The therapy of osteomyelitis includes surgical debridement, appropriate antibiotics, and adequate nutrition. Appropriate management of the dead space created at debridement surgery is mandatory in order to arrest the disease and maintain the integrity of the skeletal part. The goal of dead space management is to replace dead bone and scar tissue with durable vascularized tissue.[10] For this reason, secondary intention healing is discouraged since the scar tissue that fills the defect may later become avascular. Suction irrigation systems are not recommended because of the hight incidence of associated nosocomial infections and the unreliability of these setups.[11,12] Complete wound closure should be attained whenever possible. Local tissue flaps or free flaps may be used to fill dead space.[13,14] An alternative technique is to place cancellous bone grafts beneath local or transferred tissues where structural augmentation is necessary. Careful pre-operative planning is crucial to make efficient use of the patient's limited cancellous bone reserves. Open cancellous grafts without soft tissue coverage are useful when a free tissue transfer is not a treatment option and local tissue flaps are inadequate.[15] Finally, if movement is present at the site of infection, measures must be taken to achieve permanent stability of the skeletal unit. Local antibiotic delivery systems are increasingly being used to sterilize and temporarily maintain a dead space.[8,16,17]

Local antibiotic delivery systems (antibiotic impregnated beads) have improved the management of complex wounds in musculoskeletal surgery. Where a thorough debridement is augmented with high sustained local antibiotic concentration, the extension of bone and soft tissue contamination to a regional infection may be prevented.[18] At the University of Texas Medical Branch (UTMB), antibiotic polymethylmethacrylate (PMMA) beads are used on the adult osteomyelitis service to sterilize and temporarily maintain dead space following debridement surgery.[8,9,16] The beads are surgically implanted in the debrided bone and covered with soft tissue. Serum, inflammatory fluid, and antibiotic collects in the space around the beads, termed seroma. The PMMA beads are left in place for three to four weeks and are then surgically removed and usually replaced by bone graft.[8,18]

The antibiotic(s) used in the beads should provide seroma concentrations above the breakpoint sensitivities for three to four weeks and should yield adequate concentrations in both granulation tissue and bone. The bead antibiotic should not produce toxic serum concentrations. At present, antibiotics selected for bead delivery must be in powdered form and are selected to correspond to the sensitivities of the wound pathogens. At UTMB, the most commonly used hand–made antibiotic impregnated beads are clindamycin, tobramycin, and vancomycin. There are several inherent disadvantages to using PMMA beads. These include the necessity of additional surgery for bead removal and failure of all the antibiotic to be eluted from the PMMA bead by four weeks.[16] Biodegradable beads would have the distinct advantage of releasing all the bead antibiotic and eliminating the need for surgical removal of the beads. At UTMB an *in vitro* study was performed to compare the efficacy of PMMA beads with biodegradable beads.

Polymethylmethacrylate, 2000 molecular weight poly–lactic acid (PLA), poly–(DL–lactide)–co–glycolide (90:10, 80:20 and 70:30 PLCG) and a combination made of 2000 molecular weight PLA and 70:30 PLCG were individually mixed with clindamycin (antibiotic:power ratio of 1:6.6), tobramycin (1:4.1) or vancomycin (1:10.0). Monomethylmethacrylate was added to the PMMA mixtures and methylene chloride to the PLA and PLCG mixtures and stirred. Three beads of a comparative size (eight millimeters) of each mixture were made and allowed to dry. Each bead was placed in one milliliter of phosphate buffered saline, pH 7.2 and incubated at 37°C. The phosphate

buffered saline was changed daily and the samples stored at –70°C. The antibiotic concentration in each sample was then determined by a microbiological diffusion assay.[20] Standard two–fold serial dilutions were made for all the antibiotics using phosphate buffered saline as the diluent for the standards. The diameter of the zones of inhibition for each standard was measured and meaned. The means were plotted onto semilog paper to prepare a standard curve. The unknown antibiotic concentration of the samples were determined by comparing their respective zone size means to the standard curve.

All the beads demonstrated high concentrations at day one (300 to 12,000 μg/ml). PMMA beads with tobramycin and clindamycin had concentrations well above break-point sensitivity concentrations for more than 90 days but vancomycin concentrations dropped by day 12. Biodegradable beads had a weight increase before they disintegrated and dissolved. The 2000 molecular weight beads antibiotic concentrations dropped rapidly, but they remained effective for 38 days (clindamycin), 49 days (vancomycin) and 65 days (tobramycin) and dissolved in 100, 120 and 80 days, respectively. The 80:20–beads released effective concentrations of vancomycin (41 days) and clindamycin (48 days) and dissolved that day. Tobramycin dissolved later (day 80), but released effective concentrations for 51 days. The 70:30 beads showed the fastest degradation, dissolving by day 36 (vancomycin), day 37(clindamycin), day 38 (tobramycin) while releasing effective antibiotic concentrations above 100 μg/ml up to day 27, and remained effective for 35 days (vancomycin),36 days (tobramycin), 37 days (clindamycin) before dissolving by day 40.

In summary, low molecular weight PLA and PLCG beads released high concentrations of all the antibiotics tested *in vitro* for the period of time needed to treat bone infections. PLA and PLCG beads are superior to PMMA beads in their release of vancomycin and they are biodegradable. Additional *vitro* and *in vivo* research is needed to determine more predictable and accurate degradation rates and to explore possible utilization of these materials in orthopaedic surgery. Antibiotic impregnated biodegradable beads have a potential role in the prevention and management of musculoskeletal infections.

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TABLE 1 - CIERNY AND MADER CLASSIFICATION SYSTEM

Anatomic Type

Stage 1-   Medullary osteomyelitis
Stage 2-   Superficial osteomyelitis
Stage 3-   Localized osteomyelitis
Stage 4-   Diffuse osteomyelitis

Physiologic Class

A Host-   Normal host
B Host-   Systemic compromise   (Bs)
          Local compromise   (Bl)
C Host-   Treatment worse than the disease

TABLE 2 – SYSTEMIC OR LOCAL FACTORS THAT EFFECT IMMUNE SURVEILLANCE, METABOLISM AND LOCAL VASCULARITY

Systemic (Bs)	Local (Bl)
Malnutrition	Chronic lymphedema
Renal, liver failure	Venous stasis
Diabetes mellitus	Major vessel compromise
Chronic hypoxia	Arteritis
Immune deficency	Extensive scarring
Malignancy	Radiation fibrosis
Extremes of age	Small vessel disease
Immunosupression	Complete loss of local sensation
Tobacco abuse	

Cierny and Mader Classification System

ADULT OSTEOMYELITIS
TREATMENT PROTOCOL (1991)

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During the last nine years, over 700 patients with refractory long bone infections have entered this prospective study to assess the anatomic and physiologic parameters of adult osteomyelitis. The treatment format has remained unchanged. Patient selection and medical/surgical treatment are predetermined by a clinical staging system based on four factors: the site of infection, the extent of necrosis, the condition of the host, and patient disability.

In 1983, antibiotic beads were added to the management options. Shortly thereafter, we changed from an empiric six weeks of systemic therapy to six, four, two or one week(s) based on the clinical stage and method of dead space management. Bone transportations and distraction histogenesis methodologies added a new dimension to

Cierny

our dead space management in 1988. Corticotomies are performed at the 2nd debridement. The universal treatment algorithm (seen above) combines the formats for all stage entries, includes amputations, and schedules the management of treatment failures.

I. PATIENT SELECTION

A) Disease Evaluation

   1) History: surgeries, complications, etc.

   2) Physical exam: sinus tracts, scars, condition of the extremity, reconstruction options

   3) X–ray findings: plain films, tomograms, CT scans, MRI scans

   4) Nuclear scans: Technetium, Galium, Indium

B) Patient Evaluation

   1) Complete history and physical examination

   2) Laboratory profiles:

      hematopoietic

      metabolic

      immunologic

      nutritional

   3) Outpatient testing:

      transcutaneous oxygen levels

      arteriogram

      *organism sensitivities (Bχ)

   4) Disability Assessment

      (present condition vs. prognosis for cure)

C) Clinical Staging of Disease and Host

   1) **Anatomic Type:**

      Type I: medullary osteomyelitis

      Type II: superficial osteomyelitis

*See Section III B

14

Type III: localized osteomyelitis

Type IV: diffuse osteomyelitis

2) **Physiologic Class:**

A–host: normal systemic defenses,

        metabolic capabilities

        vascularity

B–host: local (L), systemic (S), or

        combined (S, L) wound healing deficiencies

C–host: -minimal disability (present)

        -high treatment morbidity (anticipated)

        -poor prognosis for cure

3) **Clinical Stage:** (Type + Class = Stage)

Infected non–union

    Tibia

    +   = IVB(L) Osteomyelitis

    Radiation

    Fibrosis

719 TREATMENT CANDIDATES

(1981 – 1990)

Stage	No.	Stage	No.
IA	14	IIIA	82
IB	28	IIIB	35
IIA	30	IVA	201
IIB	71	IVB	258

II.   EARLY PATHOGEN IDENTIFICATION

A)   Specimens obtained prior to debridement.

B)   Culture based on biopsies from deep tissues.

C)   Aerobic and anaerobic media utilized.

D) Histologic correlation: validated culture results and confirms pathogenesis.

E) Sequential biopsies (each procedure).

III. ANTIMICROBIAL THERAPY

A) Quantitative sensitivity testing by tube dilution technique.

B) The ideal coverage: MIC/MBC ratio 1:1 and six to eight times less than expected serum level.

* C) Pre–operative therapy: 24 hours.

D) If patient septic, administer Vancomycin and Tobramycin until culture sensitivities back

E) Hickman catheter placement at second debridement (Stages I & IV; and IIIA, B, with grafts)

F) Parenteral antibiotics:

six weeks   (IB; IVB; IIIB with grafts);

four weeks   (IA; IIIA with grafts);

two weeks   (II; III, without grafts);

one week   (amputations, open wounds)

G) Adjunctive Antibiotics – See VB3d(2) – Beads.

H) Laboratory values and antibiotic levels (when indicated).

I) Audiology monitoring (aminoglycoside therapy).

IV. HOST ALTERATION

A) Protocol:

1) patient education

2) nutritional support

3) NO SMOKING

4) pre–operative antibiotics

5) peri–operative antibiotics

6) wound protection, stabilization

*Duration determined by host and pharmacokinetic parameters (i.e., malnutrition and/or peak/trough titrations)

B)	Reverse compromising factors

## V.	SURGICAL TREATMENT

A)	**Day #0**

1)	first debridement or amputation

2)	marginal and devitalized tissue excised

3)	stabilization:

a)	prophylactic, pre–debridement (Stage I, III)

b)	initial (Stage IV)

4)	biopsy cultures from all foci encountered

5)	salvage wounds left open, amputations closed

6)	antibiotics adjusted (See IIE)

B)	**Day #5–7**

1)	Second debridement

2)	Definitive stabilization (Stage IV); corticotomy

3)	Dead Space Management

a)	open healing: Papineau technique; Ilizarov transport, etc.

b)	simple closure +/– bone grafts

c)	complex closure +/– bone grafts

d)	closure + antibiotic depot (beads, etc.)

(1)	methods: (antibiotic beads)

(a)	beads/delayed graft

(b)	beads/delayed ORIF + graft

(c)	beads + ORIF/delayed graft

(2)	systemic antibiotics s/p implantation

4 days:	A–hosts

2 week:	B–hosts

(3)	bead removal:

        (a) without graft = 7–10 days

          (simple dead space)

        (b) graft exchange at 4 weeks

        (c) perioperative antibiotics delivered

          24 hours before/after exchange

  4. Hickman catheter

At the second look debridement a clean, alive wound is confirmed. If no further excision is necessary, reconstruction is completed whenever possible, safe, and practical. Open healing by secondary intention is avoided for all but small, shallow wounds as the failure rates for large, deep wounds are unacceptable (35–40%); rated for deadspace managed with perfusion or suction–irrigation are similar (25–30%).

Open cancellous bone grafting with autogenous materials improves the host's biologic response and success rate for A–hosts (85%) but is not recommended for patients with wound healing deficiencies (20%). When the wound can be closed with local or transferred, perfused tissue, the results of cancellous (autogenous) grafting are 94% and 80% for A–hosts and B–hosts, respectively. Marrow–free, freeze–dried, cancellous allografts are used to expand the graft volume by no more than 33%.

Free bone transfers (fibula, ilium, scapula) are difficult to perform and apply mostly to segmental osseous defects with hard and soft tissue losses. The high patient morbidity and low success rates (65–74%) make bone transportation (Ilizarov, etc.) an attractive alternative.

443 CASE STUDIES
(Success rates at 2 years)

IA	100%	IB	95%
IIB	100%	IIB	77%
IIIA	98 %	IIIB	94%
IVA	99%	IVB	87%

99%　　　　　87%

Overall = 92%

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THE CURRENT STATUS OF BONE AND CARTILAGE ALLOGRAFTING

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INTRODUCTION

Even prior to the earliest reports by Lexer near the turn of the current century[25,26] orthopaedists were intrigued by the thought of allograft implantation for massive defects in the skeleton. Early in the 1960s following the discovery of Herndon, Chase and Curtiss[7,22] that freezing reduced the immune response, numerous attempts to allograft implantation were reported principally by Ottolenghi in Argentina,[37] Volkov in the USSR[45] and Parris in the U. S.[38,39] and all showed considerable promise; but also a rather discouraging unpredictability of result probably on the basis of the immune response. Massive custom metallic implants entered the field of Orthopaedics at approximately the same time[2,5,12,24,30] and these techniques along with segmental replacements using fibular autografts[15,16] appeared to replace the use of allogeneic materials in the hands of the tumor surgeon for approximately a decade or more.

Over the last few decades as surgeons became more daring in limb sparing procedures[9,10 12,23,36,41] and based on the use of adjuvant chemotherapy, patients began to outlive their sarcomas,[13,21,27,40,46] it became apparent that the autograft segments were limited by the amount of available bone and that the custom metallic implants did not "hold up" as well as anticipated. Attention was once again brought to bear on the allograft system and investigative efforts were directed at improving the methods of implantation,[9,10,23,29,35,36] ameliorating the effect of the immune response (or at least defining it)[4,8,18] and making banking safe and more competent at filling the needs of the surgeon performing limb sparing survery.[6,11,43,44]

Since 1971, the Orthopaedic Oncology Unit at the Massachusetts General Hospital (MGH) has been performing allograft implantation using fresh frozen cadaveric allogeneic segments in which cartilage has been cryopreserved with glycerol or DMSO prior to freezing. To date, we have performed 638 such procedures mostly for parients with aggressive or malignant bone tumors and have from time to time reported on not only the end results of these procedures[23,31,32,33,34] but on the major complications,[1,28,44] banking procedures.[6,11,42,43,44] This current report is a comprehensive update of these data for the clinical series; and based on these studies, an attempt to define some approaches to future management using the allograft system.

BACKGROUND

Ample evidence exists that allograft bone, even when frozen excites an immune response in the host tissues which is variable in extent[4,8,18] and effect on the operative procedure.[19] Responses vary in the experimental systems from rapid dissolution of the graft (fortunately rare in humans) to "walling off" of the segment with almost no vascular invasion (more common but still less than 10% in our and other series).[19,32,36] It is further believed that the infection rate (much higher than one would expect for even this type of surgery) is a manifestation of the immune response along with fractures, which represent the two major complications of the procedure and account for most of the failures.[1,28,31,35,44]

In similar fashion, cartilage is known to be antigenic and in fact has been shown to evoke a profound cellular and humoral antibody response.[8,18] The issue with cartilage is the possibility that the pore size of the matrix of cartilage (less than 80 Angstroms) will be unlikely to allow the egress of antigen or the ingress of either cells or antibody so that as long as the matrix is intact the cartilage remains "immunologically privileged".[8,18] Cryopreservation with DMSO seems to help reduce the likelihood of cartilage destruction (especially autolytic lysosomal enzyme activity) although even the most rigorous of techniques has thus far been unsuccessful in maintaining more than 50% of the cells viable.[42]

Of considerable importance in such a procedure is the maintenance of a competent and safe bank with an inventory of sufficient size so as to offer optimal sizing for the replacement part.[43] The MGH Bone Bank established in 1974 has a set of guidelines

which help to guarantee that the parts are disease free, appropriate in shape and of proper size.[11,43] All harvests are performed under sterile conditions in the Operating Room. Donors are screened carefully for absence of malignancy and infection and the donor and the individual parts tested for bacterial and viral contamination.[3,43] The cartilage is preserved in DMSO, and parts placed in glass sterilized plastic bags, x-rayed in two planes and frozen to -80° C. Thaw occurs in the OR and is achieved rapidly by immersion in warm Ringer's lactate (60° C.).

THE PATIENT SERIES

From November 24, 1971, until April 1, 1991, the Orthopaedic Oncology Service at the Massachusetts General Hospital has performed 638 allograft transplants including 43 in the pelvis. Because the problems are different for the pelvic grafts, for this presentation we will review only the 595 such procedures which were performed in extremities most of which were done for malignant of aggressive primary or metastatic tumors of bone. Two hundred and seventy-six (46%) of the patients were male and 319 (54%) were female. The followup was from 1 to 233 months with a mean value of 55 months; the average age for the patients was 32.2, with a range from 4 to 80 years.

The operative procedure conformed to principles of management of benign and malignant bone tumors and for the most part (82% of the 595 cases) were classified as either marginal (225/595) or wide (265/595) resections of the tumor containing bony part.[14,17] Some of the patients received chemotherapy or radiation and virtually all had had at least one prior procedure and some had had several.

The diagnoses for which the procedures were performed are shown in Table 1 and it should be evident that a considerable number of the patients are Stage 0 (114) and Stage 1 (269).[14,17] True high-grade malignancy (Stage 2 or Stage 3) accounts for 212 of the 595 patients (35.6%)

The anatomical sites for the transplants are shown in Table 2 and as can be noted, 326 (55%) of the grafts are osteoarticular (mostly femur, tibia, humerus, and radius); 131 are intercalary (22%) (mostly tibia, femur, and humerus); 78 are allograft and a prosthesis (13%) (mostly the hip or knee) ; and the remaining 60 (10%) are allograft arthrodeses (mostly about the knee or shoulder).

RESULTS

Four-hundred and eighty patients in the series have been followed for two or more years and hence could be utilized for review of the results of the procedure. The mean length of continuous followup for this group of patients (patients who died or were lost to followup were censored) was over 65 months ranging from 24 to 209 months. The patients were seen regularly and studied for evidence of local recurrence or complications of the procedure. They were then scored as **excellent** (no evident disease, NED), return to virtually full function of the part without pain or significant disability; **good** (NED, modest to moderate limitation of function, no pain or major disability); **fair** (NED, major limitations with a brace or support such as crutches, cane, etc., required) some tolerable pain; and **failure** (dead as a direct consequence of local recurrence, amputation of the part of removal of the graft for recurrence or complication).

Using such a scoring system (which compensates for comparisons of various anatomical regions) the end results at the current time are shown in Table 3. As can be noted, the 266 osteoarticul: ʳgrafts show a 73% excellent or good result with 27% graded as fair of failure. The 104 intercalary grafᵗ⸴ ᶠared better with 88% currently graded acceptable while the allograft prostheses group are intermediate (81% excellent or good). The allograft arthrodeses are the poorest group with only 4% graded as excellent and 55% graded as good. The overall score for the series of 480 patients show a figure for "acceptable" (excellent or good) results at 75% but if the 25 tumor failures are deleted from the series, that value climbes to 80% (43% excellent and 37% good).

Perhaps a better measure of the procedure is the numbers of complications. These are shown in Table 4. As can be easily noted, 155 of the 480 patients are high grade tumors (Stage 2 or Stage 3) and hence subject to major tumor complications. A 25% percent death rate, 41% metastasis rate and 12% recurrences are not inconsistent with results obtained for any series of limb sparing procedures for high grade tumors followed this long. Further, it should be noted that 25 of these 155 patients had Stage 3 disease prior to surgery and 10 of these were metastatic carcinoma. The rates for death (13/25), metastasis (25/25) and local recurrence (4/25) for these patients is higher than the overall group and influence the values obtained for the others. If just the 130 Stage 2 patients are studied, the death rate is 20%, the metastasis rate is 36.2% and the local recurrence rate 10.8%.

In terms of allograft complications as shown in Table 4, these are higher than one would like, especially infection which not only includes 10% of the patients but accounts for 39% (47/104) of the failures. Fractures are also high in number (89/480; 19%) but are slightly less pernicious accounting for only 25% of the failures. Although non-union occurs with considerable frequency in this series (14%), the effect was for less devastating in that only 2.7% of the failures were in any way associated with this complication. Unstable joints only occurred in 7% of the 323 patients at risk (allograft arthrodeses and intercalary grafts are not at risk) and played a significant role in only 4 of the failures.

Reoperations were plentiful in this group, but reflected not only the allograft complications but the not-so-occasional procedure at the same antomical site or other sites as necessitated by tumor management. Of the 480 patients, 278 (58%) did not require additional surgery, but 140 (29%) had one and 62 (12%) had two to four additional operative procedures.

The ultimate analytic tool for a series of cases such as this is the Kaplan-Meier Life Table analysis and Cox regression system, both of which demonstrate that only infection, fracture, recurrence, the use of chemotherapy and stage had a significant impact on results. Furthermore, Kaplan-Meier plots show that most of the failures (both allograft and tumors) occur by three years and that the curve declines little after that point. These data strongly suggest that once the problems of infection (almost all appear by 7 months),[28,44] fracture (most of which occur by three years)[1] are no longer issues, the graft becomes "Stable" and lasts at least through the 15 or so years of additional observation afforded by this analysis. Kaplan-Meier plots showing the overall curve (Figure 1), the effect of infection (Figure 2), fracture (Figure 3).

DISCUSSION

From the data presented above, it is apparent that in our series as well as in those from other clinical units, massive allografts are an effective method of dealing with connective tissue tumors and other aggressive or some benign conditions affecting the skeleton. From the data reported, it seems evident that fully 80 percent of the patients do reasonably well and that this value varies to some extent with the type of graft, the anatomical site and the stage of the lesion (and hence complexity of the surgery and relative necessity for chemotherapy). The three principal factors which appear to most

significantly affect the end results are recurrence, infection and fracture; and, together these account for over 85% of the failures. Of considerable importance in analysing these data is the apparent fact from study of Figure 1 that the failure rate is highest in the first year and then tails off rather rapidly. Few failures occur after the third year suggesting that the grafts establish an equilibrium state with the host, possibly not getting any better in terms of function over the years, but more importantly not getting any worse. This projection is the antithesis of the experience of the patients with metallic fixation where five- and ten-year failure rates show considerable and progressively greater increments over the rates reported for the early periods.[20]

The crucial question is: Can these results be improved? If one accepts the thesis that most of the complications are immunologically directed (and hence represent a "rejection"), the logical approach to the problem is to improve the results by either immunosuppression or better matching of the donor and host. The former is difficult to justify for two reasons. The first and most obvious is that the immunosuppressive agents have a mortality rate of their own. Thus, one finds oneself in the awkward role of advocating a life-threatening drug for a limb-threatening disease. Furthermore, treating a patient with a high-grade sarcoma with an immunosupressive drug and thus interfering with the immune system is likely to increase the rate of growth or dissemination of micro metastases and therefore further increase the fisk to the patient's life.

A better match is clearly advantageous for certain animal systems[8,18] and in theory would be of great advantage for humans, particularly in terms of the potential for successful implantation of vascularized grafts. The issue which faces us at least in theory is that a perfect match may not really be desireable since such a graft is likely to undergo the devastating changes seen in the osteoarticular form of osteonecrosis of bone (only rarely seen in frozen cadaveric allogeneic implants). The second problem with matching is a practical one. It would seem to be very difficult to match not only for size and shape (believed to be essential to achieve good results!) and also histocompatibility complex. A recent unpublished preliminary study showed that either pre-or post-transplant sensitization of class 2 antigens appeared to augur poorly for the grafting procedure.[19] If these data are confirmed by longer observation of the

series, it should be possible with large enough banks to obtain such a limited match and thus improve results.

Additional ways to improve the current results would include further attention to DMSO cryopreservation of the cartilage. Currently the best obtainable result is about 50% viability for *ex-vivo* intact cartilage segments [42] (in sharp contrast with the almost 100% viability achievable by freezing and thawing matrix free cells in culture with the same concentration of DMSO).[42] These data support the contention that the passage of DMSO through the matrix to each the cell is not free and will require some special techniques. In another area, improved attention to the internal fixation devices might result in fewer fractures and better use of plastic surgical procedures and particularly free or pedicled muscle flaps will and have already materially reduced skin slough and infection rates (our rate for calendar year 1989 is 5.3% and for 1990 is 3.0% compared with the rates for the analogous years of the prior decade of 7.1 and 14.2 respectively).

It is evident that the system remains imperfect and that untoward events such as infection, fracture and non-union make the outcome unpredictable and at times lead to failure. It should be apparent however that failure is relative particularly in that 79 patients whose grafts failued for allograft complications (rather than for tumor) over 75% were salvaged by subsequent surgery. The amputation rate for non-tumor related complications for the entire series of 480 patients is less than 5% and even adding the tumor failures in brings that value to less than 7%.

Research continues in a number of areas as outlined above. A major breakthrough might be accomplished by reduction in the immune response and improved networking in banking so that a greater number of potential grafts are available for each patient. Clearly improvement in surgical technique continues to make the operative procedure more predictable and safer for the patient. Ultimately it is hoped that a sufficiently predictable and high rate of success can be achieved to encourage surgeons to add this procedure to their armamentarium for the management of lesions other than malignant tumors and massive loss.

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TABLE 1 – ALLOGRAFT TRANSPLANTATION DIAGNOSES
FOR 595 PATIENTS 11/24/71 TO 03/23/91

TUMORS

Giant Cell Tumor	123
Central Osteosarcoma	113
Chondrosarcoma	90
Parsoteal Osteosarcoma	32
Fibrosarcoma or MFH	22
Adamantinoma	17
Ewings Sarcoma	18
Metastatic Carcinoma	16
Osteoblastoma	10
Soft Tissue Tumors	10
Chrondroblastoma	7
Desmoplastic Fibroma	6
Angiosarcoma	5
Lymphoma	3
Chondromyxoid Fibroma	3
Aneurysmal Bone Cyst	3
Myeloma	2
Osteochondroma	2
Leiomyosarcoma	3
Osteoid Osteoma	1
Liposarcoma	1

NONTUMEROUS CONDITIONS

Failed Allow or TJR	53
Traumatic Loss	14
Massive Osteonecrosis	21
Fibrous Dysplasia	12
Gauchers Disease	4
Villonodular Synovitis	2
Pagets Disease	1
Eosinophilic Granuloma	1

TABLE 2 – ALLOGRAFT TRANSPLANTATION ANATOMICAL SITES
FOR 595 PROCEDURES PERFORMED
BETWEEN 11/24/71 AND 03/23/91

OSTEOARTICULAR (326)

Distal Femur	150
Proximal Tibia	72
Proximal Humerus	37
Proximal Femur	26
Distal Radius	22
Distal Humerus	12
Proximal Ulna	3
Distal Tibia	4

INTERCALARY (131)

Tibia	44
Femur	53
Humerus	25
Ulna	2
Radius	5
Fibula	2

ALLOGRAFT-PROSTESIS (78)

Proximal Femur	48
Distal Femur	19
Prixmal Ribia	6
Entire Femur	3
Entire Humerus	1

ALLOGRAFT-ARTHRODESIS (78)

Distal Femur	29
Prixmal Tibia	3
Proximal Humerus	21
Proximal Femur	4
Distal Tibia	2
Distal Radius	1

TABLE 3 – ALLOGRAFT TRANSPLANTATION RESULTS
FOR 480 PATIENTS FOLLOWED TWO OR MORE YEARS
(11/24/71 TO 03/23/89)

TYPE OF GRAFT	EXEC	GOOD	FAIR	FAIL
Osteoarticular (266)	96	99	7	64
	36%	37%	3%	24%
Intercalary (104)	74	18	1	11
	71%	17%	1%	11%
Allo Prosthesis (57)	22	24	2	9
	39%	42%	4%	16%
Allo-Arthrodesis (53)	2	29	2	20
	4%	55%	4$	38%
	194	170	12	104
Total Series (480)	40%	35%	3%	22%

IF TUMOR FAILURES (25) ARE DELETED

Grand Totals (455)	194	170	12	25
	43%	37%	3%	17%

TABLE 4 – ALLOGRAFT TRANSPLANTATION COMPLICATIONS
IN 480 CASES FOLLOWED TWO OR MORE YEARS
(11/24/71 TO 03/23/89)

TUMOR COMPLICATIONS IN 155 PATIENTS
WITH HIGH-GRADE TUMORS

Death	39 (25%)
Metastasis	63 (41%)
Recurrence	18 (12%)

ALLOGRAFT COMPLICATIONS FOR ALL 480 PROCEDURES

Infection	47 (10%)
Fracture	89 (19%)
Non-Union	65 (14%)
Unstable Joint	22 (7%)*

It should be noted that some of the patients had more than one complication so that the numbers displayed above are not additive. In fact, 227 (47%) patients had neither tumor nor allograft complications.

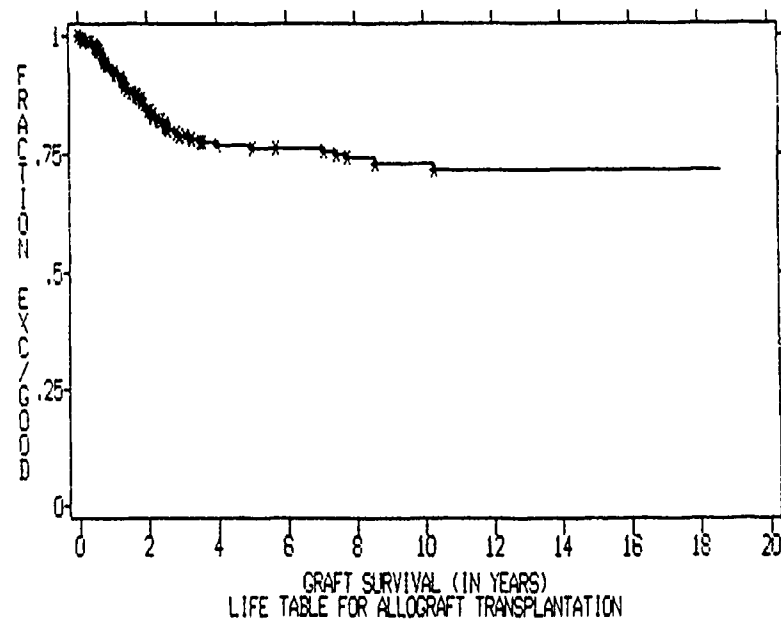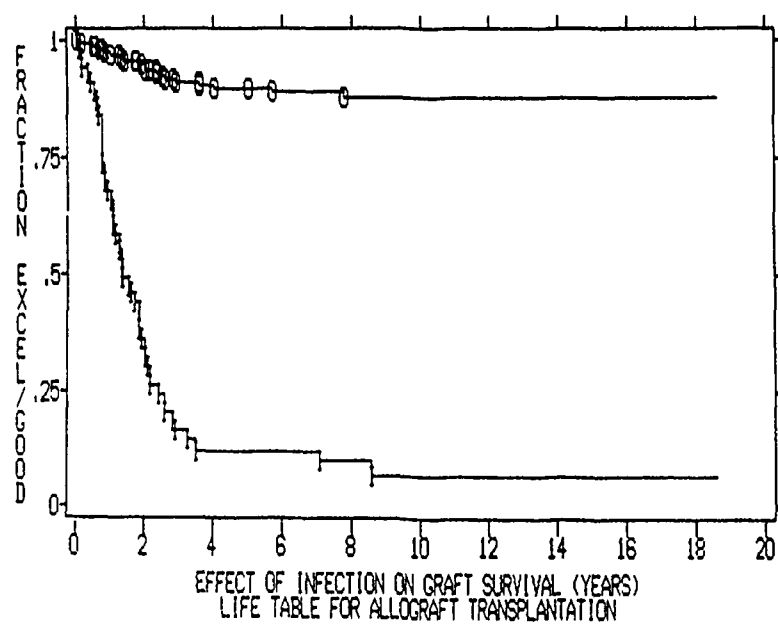*Only Ostearticular and allo-prosthesis patients are at risk for non-union.
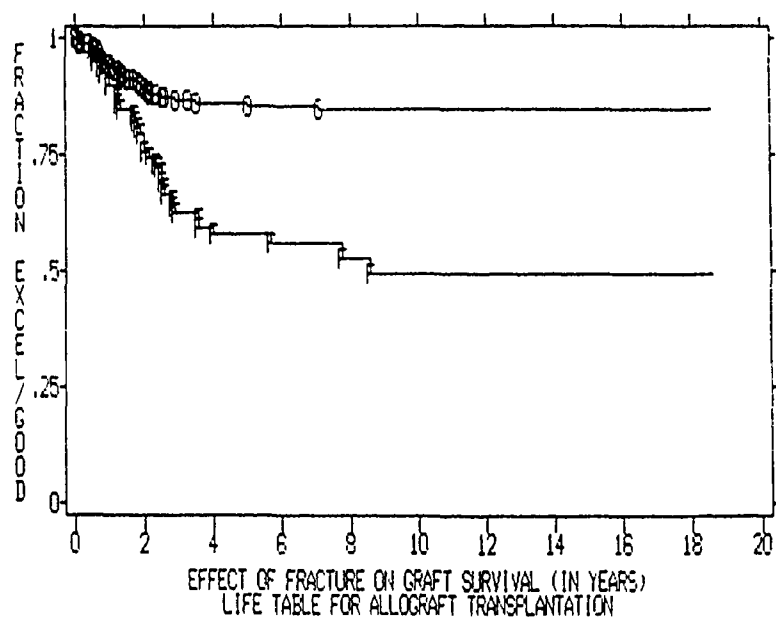
Figure 1

Figure 2

Figure 3

VASCULARIZED BONE GRAFTS IN CLINICAL SURGERY

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HISTORICAL BACKGROUND

Bone grafts are a necessary component in many reconstructive procedures performed by surgeons. Orthopaedic surgeons, maxillofacial surgeons, plastic surgeons and neurosurgeons realize that bone grafts are indispensable in their efforts to restore injured or diseased skeletal structures. Bone, next to blood, is the most frequently transplanted human tissue.

Isolated cases of clinical bone grating have been described as early as the 17th century when Job van Meekeren, a Dutch surgeon inserted a portion of a dog's skull to repair a soldier's cranium in 1668.[1] In 1820, Philips von Walter, a German, described an autograft by replacing surgically removed parts of a skull.[2] In 1880, William Macewen from Scotland described the allographic transplant of a tibia from a child with rickets to reconstruct an infected humerus in a four-year-old child.[3]

The stimulation for clinical use of bone grafting came from the simultaneous work on bone transplation of Barth[4] in Germany and Curtis[5] in the United States in the late 19th century. However, it was not until after Albee's publication of the book, "Bone Graft Surgery", in 1915[6] that bone grafting became a commonly used surgical procedure. Bone grafts are frequently employed to replace or reconstruct defects of the skeleton from infection, trauma and tumor. They are also used for arthrodesis of joints, replacement of joints, augmentation in implant revisions and congenital pseudarthrosis. The current understanding of the histologic fate of conventional (non-vascularized) autografts and allografts has not significantly changed since the earlier work of Barth and Curtis.

BONE GRAFT SOURCE

The available bone graft sources in current use are the following:

1) Autografts – non-vascularized
2) Autografts – pedicled
3) Allografts
4) Bone synthetic
5) Autografts – vascularized

AUTOGRAFTS – NON-VASCULARIZED

Fresh, non-vascularized autogenous bone grafts continue to be the standard technique for judging all other osteogenic methods. The donor graft may be cortical, cancellous, or a combination of both. The cortical graft offers the advantage or strength but delayed revascularization. The cancellous graft allows for earlier revascularization and earlier incorporation, but is inherently weaker.

Although a few osteocytes and osteoblasts may survive in the donor graft, the vast majority of the donor tissue does not survive and must be replaced by newly formed bone. This replacement of necrotic bone by new bone was initially described by Barth in 1895 as schleichender ersatz.[4] In 1914 Phemister[7] described this process of removal of dead bone and simultaneous replacement by new bone as creeping substitution. The rate of this remodeling is dependent upon the speed of revascularization to provide the primitive mesenchymal cells that differentiate into osteoblasts for the production of osteoid matrix.

The cortical bone transplant become mechanically weaker than normal bone during the early months of resorption and replacement. It may take two years before the mechanical strength becomes equal to normal bone.

PEDICLED AUTOGRAFT

The ideal bone graft is autogenous bone that remains organized and alive. Phelps in 1981 employed the concept of pedicled allograft when he connected a segment of bone from a dog as an interposition bone graft to a defect in the tibia of a child.[9] The child and the dog were connected for two weeks; however, the graft eventually failed. Huntington in 1905 described the transplantation of the fibula on its pedicle to manage large tibial defects.[10] Bone grafts which can be transferred on their pedicle without

microvascular repair are limited by the distance they can be transported. Examples include transfer of the iliac crest to the hip area, fibula to the tibia, a portion of the radius or metacarpal to the carpal bones.

ALLOGRAFTS

When massive bone grafts are needed, an adequate amount of autogenous bone is frequently not available. Allografts, which have been deep frozen or freeze dried for preservation and diminished immunogenicity, are commonly used for bone replacement. These grafts are generally inferior to autografts as the repair process takes longer and replacement is less complete.

BONE SYNTHETICS

Hyroxyapatite and tricalcium phosophate are synthetic preparations that have been clinically used as graft substitutes. They are mainly used to fill cystic defects for their mechanical strength is inferior to other bone grafts.

VASCULARIZED BONE

During the last two decades, experimental and clinical studies have established that immediately vascuarlized autografts are superior to non-vascularized autografts.[11-21] In bone grafts that have immediate restoration of their blood supply, the osteocytes and osteoblasts survive. This live bone unites to the host more efficiently, retains its structural quality, size and shape better, defies resorption, and hypertrophies more than conventional bone grafts.

Significant advances in microvascular surgery during the past 15 years have made it possible to provide continuing or immediate blood flow to and from autogenous bone grafts.

With the preservation of the nutrient blood supply by the microvascular anastomosis of the nutrient vessels to the recipient vessels, the graft survives, and healing of the graft to the recipient bone is facilitated without the slow process of creeping substitution.[11,22]

Similar to the pedicle bone graft, the free vascularized bone graft is the ideal bone graft since it carries its own blood supply as well as osteoinductive, osteoconductive, and osteoprogentitor capacities. This method of bone grafting is especially beneficial

when a massive segmental bone defect is present or the surrounding vascular supply has been compromised, e.g., severe trauma or irradiation. Of course, the main disadvantages are the greater technical difficulty and length of the proceure.

DONOR SITES FOR VASCULARIZED GRAFTS

The major donor sites for free bone transfers are the following:

1) fibula

2.) iliac crest

3) rib

All have a vascular pedicle of sufficient length and caliber. They can be transferred alone or as a composite with skin and muscle. The fibula is the most universal donor because of its length, strength and large size of nutrient vessels (peroneal).

The iliac crest provides both cortical and cancellous bone as well as over-lying soft tissue composite. However, the defect is large, often mutiliating, the dissection is difficult, and the length and strength of bone is not comparable to the fibula. Its curvature also restricts its use to defects 10 centimeters or less.

The rib is certainly expendable and its pliability makes it desirable in some plastic procedures, e.g., mandibular reconstruction. Its inherent weakness limits its usefulness.

Lesser used donor bone for free transfers include the following:

1) scapular

2) radius

3) ulna

4) metacarpal

5) metatarsal

6) humerus

7) tibia

PREREQUISITES FOR VASCULARIZED BONE TRANSFER

Since this procedure is more complex, lengthy, and requires an experienced microvascular team, it should be reserved for those procedures where traditional methods usually are unsuccessful. It's especially suited for long bone defects greater than six centimeters.

An experienced microsurgical team proficient in free tissue transfers must be available for lengthy procedures that may encompass six to ten hours. The vascular anatomy of the recipient site must be thoroughly studied to be certain that the vessels are adequate, not only to receive the nutrient vessels of the donor, but also continue to supply the adjacent and more distal structures. In about every instance, I obtain an arteriogram of the recipient field. If the fibular is to serve as the donor bone (over 90 percent of my cases) I obtain an arteriogram of the donor area. This preoperative planning provides additional information which gives added confidence to the surgeon harvesting the fibula. However, in our more than 400 arteriograms on the vascular supply of the leg, we have found an abnormal vascular pattern to the fibular area in less than five percent of the patients.

The donor site morbidity from harvesting the fibula is low in our experience. We have reviewed 220 patients who have had their fibula harvested for a vascularized fibular transfer and the overall morbidity is less than 5 percent (including motor, sensory deficient, and ankle pain).

CLINICAL APPLICATIONS

With the advances in the field of microsurgery, clinical applications of free vascularized bone grafting has been realized for the past fifteen years. We performed our first successful free vascularized fibular bone graft for non-union of a humerus in an adult male in 1974. Taylor, Miller and Ham[23] first reported the clinical use of a free vascularized fibular bone graft in two cases of traumatic segmental bone loss in a tibia. Clinical experience during the last one and a half decades has clearly demonstrated that vascularized bone transfers decrease the time of union and immobilization necessary to reconstruct large defects in bone. These advantages are especially evident when the diaphyseal segmental loss is greater than six centimeters.[11,16,24,25] There is increasing evidence that vascularized bone grafts are beneficial in the treatment of avascular necrosis of some bony structures.[26-32]

When a large soft tissue defect occurs in conjunction with the large bone gap, skin and subcutaneous coverage can be accomplished by two major methods: 1) single stage osteocutaneous flap or 2) a cutaneous or myocutaneous flap followed by the vascularized bone graft.

45

The vascular anastomosis may be either end-to-end or end-to-side, whichever is easier. Both venous and arterial anastomoses are necessary for graft survival. Interposition vein grafts are utilized when needed.

Currently the more common uses of vascularized bone grafts include the following:

1) Segmental bone loss

    a) trauma

    b) tumor

2) Nonunion

3) Chronic osteomyelitis

4) Congenital pseudarthrosis

5) Avascular necrosis

SEGMENTAL BONE LOSS

Free vascularized bone transfer is an excellent method for reconstruc '' · ' ;e segmental bone loss from trauma. Bone tumors, especially benign or low grade malignancies, may be managed by *en bloc* or radial bone resection and reconstruction by free vascularized bone grafts. Bone stabilization may be obtained by plates and screws or external fixators. Currently we prefer plates and screws, but care must be used to prevent injury to the nutrient vessels.

NONUNION

The preferential treatment at our institution for chronic non-unions with a large gap is a vascularized bone graft. However, this excellent method is being challenged by the Ilizarov local bone transport method.[3] Supplemental conventional cancellous or corticocancellous bone is usually added to the area.

CHRONIC OSTEOMYELITIS

Vascularized bone segment transfers are also employed in the management of chronic osteomyelitis. The most important segment of the treatment is the thorough debridement which often requires more than one procedure to excise the involved bone and surrounding structures. The type of donor tissue, i.e., the particular bone with or without muscle and/or skin, depends on the size and location of the defect, the surrounding environment, and the surgeon's experience.

CONGENITAL PSEUDARTHROSIS

Treatment of congenital pseudarthrosis of the tibia continues to be an orthopaedic dilemma. Bracing, internal fixation, massive bone grafting and electrical stimulation all experience a high failure rate. Several authors have reported success rates of approximately 90% using a free vascularized fibula.[34-36] Our limited experience with this technique in congenital pseudarthrosis of the tibia and radius has been encouraging and the success is similar to those reported previously.

AVASCULAR NECROSIS

Avascular necrosis of bone most commonly occurs in the femoral head. There are numerous causes, but the most common are related to alcohol, steroids, trauma, and collagen vascular disease. In at least one-fourth of the patients, the etiology remains obscure.

The goal in the management of avascular necrosis of the femoral head should be to preserve rather than replace the femoral head. Many methods of treatment have been proposed but none have been completely successful. Since 1979, we have attempted to treat this problem with a vascularized fibular graft using microvascular techniques.

Three hundred and twenty-seven patients have been treated from 1979 through May, 1991, all symptomatic preoperative and all demonstrating x-ray changes of avascular necrosis. The surgical technique is performed with the patient in the lateral position. One team harvests five inches of fibula with a four to six centimeter pedicle of peroneal artery and vein. (Figure 1) A second team exposes the hip joint with a lateral incision through the tensor fascia lata and the gluteus medius. (Figure 2) The lateral femoral circumflex artery and vein are located with receipient vessels. Using a C-arm for intraoperative x-rays, a large hole is drilled through the femoral neck. Specially enlarged drill bits are used to make a core large enough (usually 19 millimeters in diameter) to prevent compression of the fibular graft with its vascular pedicle. Using a custom designed speed drill with a head size of 12 to 17 millimeters all of the avascular bone is removed and the subchondral bone is visualized with loupe magnification. Cancellous bone from the proximal femur and greater trochanteric region is packed into the area of the excised necrotic bone. The fibula with its vascular pedicle is inserted and fixed with a small Steinmann pin to prevent retrograde migration. The vascular anastomosis is then completed (Figure 3).

47

Postoperative management includes a digital arteriogram at five days to verify that the bone graft remains vascularized. The patient is kept touchdown weight bearing with crutches for six to nine months. Magnetic resonance imaging is helpful in evaluation of patients preoperatively and postoperatively.

We use the six stage classification scheme of avascular necrosis described by Enneking.[39] Although 190 of our 327 patients have been in Stage III or IV preoperatively, we would prefer to do this procedure prior to any femoral head collapse (Stage I or III). Our patients are young, however, 30 percent were 28 years of age or less (average 34 years) and no other acceptable procedure could be recommended.

Of the 85 patients that have been followed for five years, twenty-one of those patients have had additional surgery. Donor site morbidity has occurred in five patients and weakness of the EHL in five patients.

Of the 327 free vascularized fibular grafts done, 41 have had to be converted to a total hip replacement.

Rationale for use of the vascularized fibular graft to avascular necrosis of the femoral head is fivefold. It adds bone, decompresses the femoral head, removes dead bone, adds a vascular pedicle, and allows the patient six to nine months of non-weight bearing ambulation. The procedure is lengthy, early results are encouraging, and long-term follow-up is needed.

Treatment of avascular necrosis of other bones such as the lunate and talus by vascular bundle or vascular bone transfers have been described, but the numbers are small.

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FIGURE LEGEND

Figure 1

Donor fibula with peroneal artery and veins is harvested from ipsilateral leg to be inserted in core in femoral neck and head.

Figure 2

Surgical approach to femoral neck and head is between gluteus medius and tensor fascia lata. Tarnsverse branches of lateral femoral circumflex artery vein are used as recipient vessels for vascularized fibular graft. Large core is made in femoral neck to area of necrotic bone in femoral head. (Reprinted with permission from Urbaniak, J. R.: Aseptic necrosis of the femoral head treated by bascularized fibular graft. In Urbaniak, J. R. (ed.) Microsurgery for Major Limb Reconstruction. St. Louis, C. V. Mosby, p. 180, 1987.)

Figure 3

All necrotic bone is removed from femoral head by operating through core using high-speed burr, headlights, and magnification. Cancellous bone chips and vascularized fibula are inserted. Peroneal vessels from the donor fibula are anastomosed to the lateral femoral circumflex vessels.
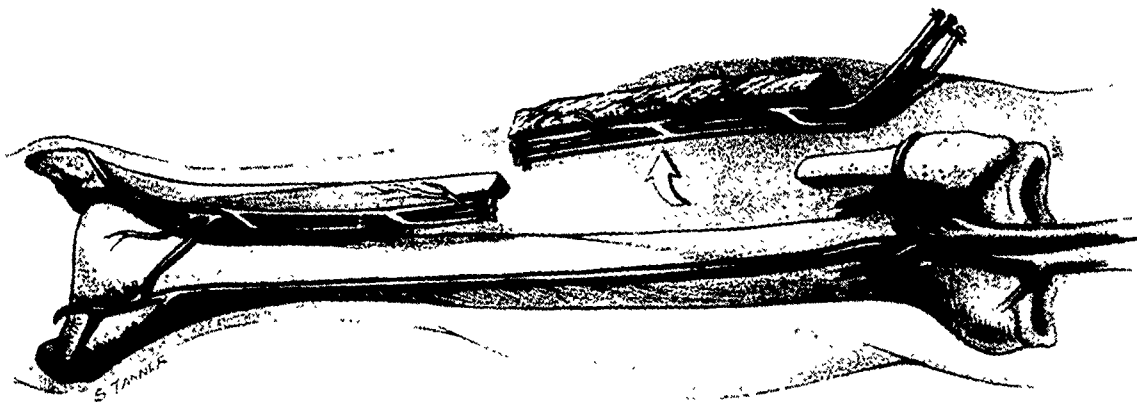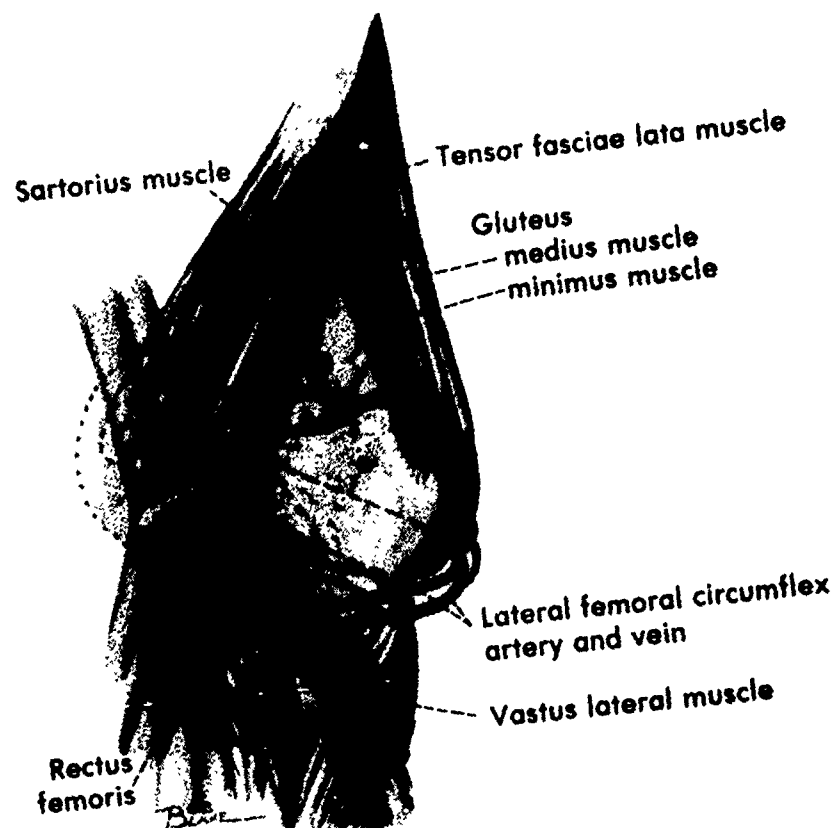
Figure 1

Sartorius muscle

Tensor fasciae lata muscle

Gluteus
medius muscle
minimus muscle

Lateral femoral circumflex
artery and vein

Vastus lateral muscle

Rectus
femoris

Figure 2

Figure 3

THE ILIZAROV TECHNIQUE: A METHOD
TO REGENERATE BONE AND SOFT TISSUE

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In 1951 Professor Gavril Abramovich Ilizarov from Kurgan in the Soviet Union developed a circular external fixator for the treatment of fractures (Figure 1). Over the ensuing years he discovered the techniques of physeal distraction, corticotomy lengthening, bone transport, and many others. The common basis for all of these methods he called the theory of tension stress.[1,2] Through controlled mechanically applied tension stress, Professor Ilizarov was able to show that bone and soft tissue can be made to regenerate in a controlled reliable and reproducible manner. His experimental work on tissue regeneration under distraction lead to his doctorate thesis in 1967. He subsequently established the Kurgan Research Institute for Experimental and Clinical Orthopaedics and Traumatology. This modern 1,000 bed hospital is divided into 18 departments, each using his method for different applications. They continue to have a very large and active research facility which conducts biochemical, biomechanic, scintographic histologic, electron microscopic, physiologic, and a variety of other types of research, all into the field of distraction histogenesis.

His methodology was first introduced to the West by Italian orthopaedic surgeons from Lecco, Italy. In North America, experience with this technique dates back to 1987.

Currently, the primary indications for the Ilizarov technique are:

1) Limb lengthening.

2) The treatment of nonunions, bone and soft tissue defects, and osteomyelitis.

3) Correction of bony deformities, joint contracture deformities, and even contour deformities of the limbs.

4) Arthrodesis.

5) Treatment of fractures and dislocations.

This article will present examples of most of these various applications as well as the scientific basis for distraction histogenesis. While the clinical experience to date has

been to the limbs, some recent research and applications of these methods to craniofacial surgery will also be discussed.

THE ILIZAROV APPARATUS

The Ilizarov apparatus is a circular external fixator that gains fixation to bone through smooth or beaded Kirschner wires of 1.5 or 1.8 mm in diameter (Figure 2). These are placed under tension and are oriented in multiple directions and multiple planes. In addition to the rings and wires, the Ilizarov system consists of multiple parts of multipurpose designation such as hinges, plates, and threaded rods (Figure 3). The frame can be assembled in an almost unlimited number of variations and combinations, depending on the task at hand. It is not surprising that this system has been labeled a human erector (mechano) set. If the appropriate construct is applied to the limb, limb segments can be moved around in space in any direction including length, rotation, angulation, and translation. In addition, the frame allows one segment of the bone to be placed under compression while another is placed under distraction. In the treatment of bone defects, intercalary segments of bone can be moved independent to the proximal and distal extremities of the bone without changing the bone length. The latter is called bone transport, a technique used to treat bone and soft tissue defects.

Unlike conventional external fixators which use threaded half pins for fixation to bone, this system uses transfixion wires. The stiffness of these wires comes from the tension that is placed across them. One end of the wire is fixed to a ring while the other end is tensioned to between 80 and 130 kg. using a dynamometric wire tensioner. Similar to a piano wire which is tensioned, the stiffness of these wires increases with increased tension.

Conventional cantilever (monolateral) external fixators utilize large diameter pins and are very stiff to bending in the plane of the pins but are much weaker in resisting bending loads 90 degrees to the plane of the pins and torsional loads. They are also very stiff to axial loading. In comparison the Ilizarov fixator is relatively stiff to bending loads but relatively flexible to axial loads.[3] Walking on the Ilizarov fixator can, therefore, be likened to walking on a trampoline where there is some give in the axial direction upon loading. As soon as the load is removed the bone segments are sprung back into place. This cyclic axial micromotion is considered to be stimulatory to bone

healing.[4] In contrast, the axially rigid constructs of the half pin fixators if left undynamized are considered to be inhibitory to bone healing.[5,6] The Ilizarov fixator, therefore, may possess some excellent biomechanical properties for bone healing. These include:

1) Shear resistance
2) Bending stiff
3) Axially dynamic
4) Circumferential stability

DISTRACTION OSTEOGENESIS

Under controlled mechanical conditions an osteotomy that is distracted apart will produce bone between the distracted bone ends. Ilizarov showed that there are a variety of factors that will affect this new bone formation. These include:[7]

1) The stability of fixation
2) The type of osteotomy used
3) The location of the osteotomy in the bone
4) The presence of a diastasis between the bone ends
5) The latency period prior to distraction
6) The rate of distraction
7) The rhythm of distraction

Ilizarov compared distraction osteogenesis under different conditions of frame stability.[1] The more stable the fixation the better was the bone regeneration. In the same experiment he also varied the type of osteotomy. He compared an open osteotomy which transected the periosteum, cortex, and endosteum to a percutaneous osteotomy which preserved most of the periosteum and endosteum to a completely closed osteoclasis in which there was no damage to periosteum and endosteum. In both of the latter two osteotomies the periosteum and endosteum remained predominantly intact. Preservation of these soft tissues decreased the rate of consolidation for the distraction osteogenesis of new bone. The completely closed osteoclasis was only slightly better than the percutaneous osteotomy. Therefore, his current recommendation is to use a percutaneous subperiosteal cortical osteotomy which we call a "corticotomy". The level of the bone cut is also a factor. The metaphyseal region is known to have a very high osteogenic potential. In comparison the diaphyseal region has a lower osteogenic

capacity. Furthermore, Professor Ilizarov claims that since the growth plate is located next to the metaphysis, the soft tissues in this region are better adapted to lengthening since they need to respond to the natural distractor, the epiphyseal plate. A metaphyseal corticotomy may, therefore, be preferable to a diaphyseal one. Any initial diastasis or translation between the bone ends may be deleterious to bone regeneration. The corticotomy should, therefore, remain closed and undisplaced. After performing the corticotomy a latency period is provided prior to distraction. A latency period of 7-10 days has been shown to be optimal in a dog experimental model.[8] In general, the two factors which relate to the latency of distraction are the age of the patient and the quality of the corticotomy. The older the patient the more time is waited prior to distraction. The latency period allows the inflammatory phase of fracture healing to subside and, thus, the distraction is begun during the reparative phase when early osteogenesis is already seen. Thus, the distraction is that of a newly formed callus. In a young child three days is a sufficient latency period, while in the older child or young adult 5-7 days is preferable. In the adult 7-14 days may be opted for. One must also consider how well the corticotomy was performed. In a minimally traumatic corticotomy, distraction may begin earlier than in a corticotomy with more significant vascular damage to the periosteum or endosteum.

Finally, two very important factors are the rate and rhythm of distraction.[2,9] The optimal rate of distraction is usually 1 mm per day. Slower rates such as 1/2 mm per day frequently lead to premature consolidation while too rapid a rate of distraction such as 2 mm per day may cause poor bone formation. The rhythm of distraction refers to the frequency of applied distraction. 1 mm per day can be applied as a single dose or can be divided into multiple doses throughout the day. One mm applied in 4 equal doses (1/4 mm four times per day) leads to more rapid bony consolidation than 1 mm applied once a day. Even greater improvement in consolidation time is seen using an automatic distractor which can apply a quasi continuous amount of lengthening throughout the day.[2] Ilizarov developed a motorized distractor which performs 60 lengthenings a day to a total of 1 mm per day. Bony consolidation under such conditions was very rapid.

The sequence, therefore, of distraction osteogenesis following the performance of a corticotomy involves:

1) Latency period

2) Distraction (Figure 4A)

3) Fixation (Figure 4B)

The distraction period is the time during which distraction between the bone ends is being performed. The fixation period is the time following distraction during which the bone formed between the bone ends is allowed to consolidate prior to removal. The apparatus can be removed once the new bone formation in the distraction gap is judged to be sufficiently strong to allow unprotected weight bearing (Figure 4C).

During the distraction period histologic and radiographic examination reveals trabeculae oriented in the direction of distraction emanating from both bone ends and terminating at a fibrous interzone which separates the proximal and distal tips of trabeculae (Figure 5). This fibrous interzone is the "pseudo growth plate" of this new bone formation. The trabeculae are conical in shape with a very wide base near the original bone end and a very narrow tip at the fibrous interzone. Detailed study of the fibrous interzone has revealed it to contain spindle shaped cells which stream into the tips of the newly formed trabeculae. Collagen formation is seen emanating from the fibrous interzone into the new trabeculae followed by deposition of mineral.[10] These spindle shaped cells from the interzone are seen to gradually differentiate into osteoblasts which produce this mineralized osteoid. They can be seen to line the outer surface of these conical trabeculae along their entire length. As one proceeds towards the base of these trabeculae, appositional new bone formation widens their bases. At the fibrous interzone no cartilage intermediary is seen between the spindle shaped cells and the osteoblasts. This process, therefore, has been termed intramembraneous ossification. Occasionally one sees small cartilage islands which have been produced from the interzone.[11] These cartilage islands are then converted to bone by the process of endochondral bone formation. These regions are felt to represent areas of increased ischemia or instability. (In rabbits this is a normal sequence of events while in higher animals it is less frequent.)[12,13] Under greater conditions of instability, fibrous tissue formation and in some cases degenerative cyst formation are seen.[12] This demonstrates the importance of stability for distraction osteogenesis formation. The interzone has been shown by vascular injection studies to be relatively hypovascular in comparison to the hypervascular trabecular regions.[12,7,10] The latter regions have cascades of

trabeculae and wide vascular channels. Between adjacent trabeculae, one can see a vascular channel. These too emanate from the relatively hypovascular interzone where the new vessels are thought to have originated. The interzone is thought to contain relatively undifferentiated mesenchymal cells which then produce bone, cartilage, fibrous, or vascular tissue.

The relative hypovascularity of the interzone is thought to reflect its volatile nature which under conditions of too rapid distraction becomes ischemic and, thus, leads to cartilage or fibrous tissue, or, cystic degeneration.

At the end of the distraction period one begins to see thickening of the trabeculae at the periphery of the new bone tube (Figure 4B). This is called neocorticalization. During the fixation phase the neocorticalization matures until the point that a sufficiently thick new cortex strong enough to resist unprotected load bearing is present. At this point the apparatus can be removed. Also during the fixation phase the fibrous interzone ossifies. After removal the regenerated bone segment continues to remodel the junction between the new and the old bone. Remodeling of the medullary trabeculae proceeds until complete recanalization of the medullary canal is seen. At the end of the process the new bone appears as a normal tube of bone identical to the host bone (Figure 4D). This is in contrast to the new bone seen in fracture healing where there is a disorganized collagen network of woven bone and the bone often never returns to its original tubular shape.[14]

Distraction osteogenesis is also seen under natural conditions. The bone growth at the perimeter of the growth plate experiences traction forces due to the attached periosteum. This new bone formation occurs in a trabecular fashion without an endochondral intermediary. The trabeculae are again oriented in the direction of the traction. Similarly, the bone seen in the sunburst appearance of periosteum elevated by a neoplasm resembles distraction osteogenesis. The tumor acts as a natural distractor to elevate the periosteum which then creates trabeculae perpendicular to the shaft of the bone in the direction of the distraction. Controlled mechanical distraction osteogenesis is a method to reproduce this natural phenomenon and to accelerate it to its maximum potential. To put matters in perspective, the human distal femoral growth plate grows approximately 50 microns per day. We perform distraction osteogenesis at 1,000 microns per day.

DISTRACTION HISTOGENESIS OF SOFT TISSUES

The mechanisms of new bone formation under controlled mechanical distraction osteogenesis as pioneered by Ilizarov are relatively well understood and have been reproduced by several investigators.[15][17] On the other hand distraction histogenesis of soft tissues is less well understood. Examples of distraction histogenesis of soft tissues are abundant in nature. A 900 fold increase in size of the female uterus under the expansion force of the fetus is an excellent example of this process. After birth there is no question that new soft tissue of the uterus and of the abdominal wall has been regenerated during the gradual distraction from within. Soft tissue distraction by a rapidly growing neoplasm employs the same mechanisms. Controlled mechanical distraction of soft tissues has been performed employing soft tissue distractors.[18] It can be assumed that the same mechanisms that are involved in soft tissue regeneration under conditions of limb lengthening are involved in these other situations. The questions that remain unanswered are:

1) Which soft tissues are amenable to distraction histogenesis?
2) Does cell proliferation exist or is this simply a stretch phenomenon?
3) Which cells undergo histogenesis under the stimulation of distraction or "tension stress"?
4) What are the optimal rate and rhythm parameters for soft tissue distraction histogenesis?

Ilizarov has investigated the effects of distraction on skin, muscle, tendon, fascia, blood vessels, lymphatic channels, and peripheral nerves.[1,2] While different tissues react in different ways, there were two predominant mechanisms at play. The first is reorganization of collagen in response to stretch and the second was neohistogenesis. For example, the initial reaction of fascia to dosed distraction was reorientation of its collagen network to stretch.[19,20] This can be likened to what happens to a net when one pulls on it. The cross hatches of the net which form square holes in the net reorient to form a diamond shaped hole and eventually just slits. After that increased fibroblastic activity was seen. Dyachkova also showed that muscle, responds by initially stretching without cell proliferation followed by a combination of stretch and a cellular response.[19] In muscle the cellular response was mixed. First there was a recruitment of cells, as evidenced by increased numbers of satellite cells. These were felt to undergo neohis-

togenesis and contribute to the growth in length of new muscle. Similar findings were reported by Appell *et al.* in athletes.[21] Second, there was addition of sarcomeres to existing muscle cells. Radiologic markers were placed on both muscle and fascia in order to determine the level at which the lengthening was occurring.[7,19,20] For the first 20% of growth and length of the muscle, the radiologic markers moved apart evenly. This implied that the muscle was lengthening evenly between its muscle tendon junctions. After 20% of lengthening of the muscle was achieved, there was greater lengthening seen at the level of the bone distraction then at other levels in the muscle. Between 20-25% lengthening, increased damage was seen in the muscle structure.[16,19,20] In a second experiment a double level bone cut was performed in the proximal and distal tibia. This time lesions were not seen in the muscle until 20-30% increase in length.[20,22] This implied that a second level of distraction can redistribute the level of lengthening more physiologically despite the increased rate of distraction to the muscle. In other Russian experiments looking at changes in muscle the first notable change was electrophysiologic.[23] This was followed by a change in histology and finally a change in the total morphology of the muscle. The recovery of the muscle occurred in reverse order. There was first a recovery of the morphology followed by the histology and only at a very late stage the electrophysiology returned to normal.

Peripheral nerves as well were seen to undergo changes under distraction.[12,24] New Schwan cells and active myelination could be seen in the peripheral nerves. A finding typical to the nerves and to many of the other tissues was observed on electron microscopy. Morphologic features typical of fetal tissue but atypical of adult tissue were observed in the nerves, muscles, and most of the soft tissues. Ilizarov claims that tension stress stimulates tissue to regress into a fetal state with the regenerate potential of fetal tissue.[1,2] If this hypothesis is proved to be correct, then tension stress could prove to be the key to unlocking secrets to limb regeneration.[25] Needless to say, distraction histogenesis of soft tissues is an area ripe for potential future research. An example of this type of research is shown in two experiments performed by Professor Ilizarov.[26] In the first he created a femur fracture with an associated vascular lesion of the femoral artery. In order to treat the vascular lesion without a bypass graft they shortened the femoral fracture by overlapping the bone ends. The arterial injury which included a segment of artery that was resected was repaired by end-to-end anastomosis after the

shortening. Three weeks after shortening the limb was relengthened without failure of the anastomosis. If relengthening was performed prior to three weeks, rupture of the anastomosis or aneurysm formation occurred. In a second experiment they created a nerve defect and treated it by resecting a segment of femur, reopposing it end-to-end, doing a primary anastomosis of the nerve ends, and then lengthening of the femur. Again, if there was at least a three week latency period the nerve remained intact at the anastomosis site. In a lecture on this subject he reported some preliminary findings that nerve regeneration under distraction was faster than nerve regeneration expected following an end-to-end anastomosis without tension. The potential for performing nerve lengthening without related bone shortening or lengthening may be a treatment consideration in the future to bridge nerve defects.

LIMB LENGTHENING

Surgical limb lengthening was first reported in 1905 by Codivilla from Italy.[18] Since that time a variety of innovative methods using different external fixation devices have been devised.[28-30] In 1963 Heinz Wagner from West Germany developed a simple monolateral fixator for limb lengthening.[31] He combined fixation with this device togeth·r with an open transperiosteal and transendosteal osteotomy followed by an immediate distraction of 1 cm between the bone ends. He then distracted at 2 mm per day. Once the lengthening was achieved an autogenous cancellous bone graft was transplanted together with stainless steel plate fixation. The external fixator was then removed. Two years after consolidation of the bone graft the plate would be removed at a third surgery. Thus, three major open surgeries were required in order to achieve limb lengthening, usually of no more than about 5 cm. Until recently, this was the standard method for limb lengthening in the Western world. Complications were the rule rather than the exception. Mosca and Moseley reported a 200% complication rate, with almost every patient having at least one serious complication and one minor complication.[32-33] In the majority of cases the goals of treatment were not achieved. Due to the plate and bone grafting, many patients had bone healing problems and malunions. Late refractures were frequent. Relative to the Ilizarov method, it is apparent that the Wagner method is unphysiologic. The osteotomy is very invasive, sacrificing both periosteum and endosteum. The level of osteotomy is in the mid ··aphysis instead of

the metaphysis and a large initial diastasis is created. There is no latency phase before distraction. The distraction rate is twice that employed by Ilizarov and at a rhythm of 1 time per day. Therefore, while Wagner's device was one step forward, his biologic technique was two steps backwards. Interestingly, the technique most popular just prior to the Wagner method, the Anderson method, employed a poor external fixator but was beginning to employ some of the biologic principles later discovered by Ilizarov for bone regeneration.[5]

Limb lengthening using the Ilizarov technique has been reported with high success rates and acceptable complication rates.[34,35] In my own reported series of 60 lengthenings, there was a 25% rate of problems that had to be dealt with in the outpatient clinic which did not interfere with treatment.[36] In addition, there was another 15% rate of what we considered obstacles which required some adjustments of the apparatus under an anesthetic. Neither the problems nor the obstacles led to any residual problem at the end of treatment. There was a 15% rate of minor complications which had very little significance which included the following: deformity <5°, transient contractures, transient sensory loss, 1 cm length loss, delayed consolidation, hematoma, and a false compartment syndrome.

There was a 25% rate of major complications which included the following: transient reflex sympathetic dystrophy, equinus contractures requiring tendoachilles lengthening, nonunion of an arthrodesis site, late bowing, and transient motor nerve palsies.

Despite all of these problems, obstacles, and complications, all of the nerve injuries fully recovered both sensory and motor deficits. The majority were not distraction related but rather related to insertion of the wires at the time of surgery. Only three of the major complications led to failure to achieve the original goals of surgery. Therefore, in contrast to the Wagner method where achieving the original goal of surgery was not usually accomplished, with the Ilizarov method the original goals of surgery are achieved in almost 100% of cases with acceptable complications rates and no permanent disability. Much larger lengthenings were possible with the Ilizarov technique and in this particular series the lengthenings ranged from 2 to 16 cm. In addition, simultaneous correction of deformity or treatment of nonunion or osteomyelitis is made possible.

The total treatment time for limb lengthening is assessed by a parameter known at the lengthening index.[7] The lengthening index is the number of months of treatment time divided by the total number of centimeters lengthened. For example, a 10 cm lengthening takes approximately 10 months. In general, a single level lengthening in a child has a lengthening index of approximately 1. The lengthening index should not be confused with the rate of distraction. To perform a 10 cm lengthening would take 100 days at 1 mm per day. This does not take into account the fixation period which is approximately two days for every day of distraction. Therefore, the lengthening index works out to be 30 days per cm or one month per cm. It is obviously preferable to decrease the lengthening index which means that the treatment time is decreased. Very few factors have been found to significantly decrease the lengthening index. Electric stimulation has been found to have no effect in an experimental model.[37] Hyperbaric oxygen has been tried with limited success in the experimental laboratory. On a more practical basis, two particular factors have been shown to decrease the lengthening index. One is to use a second level of lengthening simultaneous with the first (Figure 6).[36] Therefore, double level lengthenings for 10 cm would involve two 5 cm distraction gaps. This would be expected to lengthen and consolidate at the rate for a 5 cm gap rather than that of a 10 cm gap (Figure 7). Therefore, the lengthening index should be 1/2 month per cm. This is a significant reduction in the total treatment time, especially for large lengthenings. Another method to decrease the lengthening index is to increase the rhythm of distraction. Using an automatic distractor this has been shown both clinically and in the experimental animal to significantly decrease the treatment time.[2]

My results for lengthening in children revealed a lengthening index of 0.97 months per cm for single level lengthenings and 0.57 months per cm for double level lengthenings. On the other hand, in adults the results were 1.7 months per cm for single level lengthenings and 1.1 months per cm for double level lengthenings. In all cases the double level lengthenings significantly decreased the treatment time. However, the treatment time in adults was significantly longer than in children, as would be expected. Lengthening may be performed to all limb segments including the humerus, forearm (Figure 8), metacarpals, and digits in the upper extremity and the femur, tibia, hindfoot, and forefoot in the lower extremity.

BONE DEFORMITIES AND JOINT CONTRACTURES

The Ilizarov apparatus also has modular parts that can be used for deformity correction in any direction. The most basic deformity correction unit is the hinge (Figure 9). With an osteotomy at the level of an apex of the deformity and the hinge overlying the apex, distraction of the concavity will lead to an opening wedge correction, regenerating a wedge of new bone. If the hinge is placed away from the apex on the convex side but still at the level of the osteotomy, simultaneous lengthening and deformity correction will occur with the regeneration of a trapezoid-shaped segment of new bone. Similarly, if the hinge is placed on the concave side of the bone, compression will result from distraction to the concavity. If the hinge is placed proximal or distal to the osteotomy, translation of the bone segments will occur. In addition to hinge placement, one must insure that the construct has appropriate fulcrums and antefulcrums built into it. This uses special wires with beads on them called olive wires which act to resist the tendency of the bone to slip on the wires. Imagine trying to straighten a bent object with both hands; the immediate tendency is to put it over ones knee. The knee acts as the fulcrum while each hand acts as the antefulcrum to resist slippage of the object. Similarly, if one is trying to straighten something with both hands, the tendency is to put one's thumbs over the apex of the deformity and our index finger over the ends of the object (Figure 10). This four-point bending is similar to the Ilizarov apparatus. Olive wires are placed on either side of the apex of the deformity adjacent to the apex and an additional olive wire is placed on the opposite side of the bone at the extreme ends of the bone. This allows the apparatus to control the bone segment and together with the hinge and distraction rod on the concavity, carry out the correction (Figures 11 and 12).

Hinges are used for angular deformity corrections. When there is a rotational deformity this too can be corrected by modifying the apparatus such that the connection between one ring and the next across an osteotomy is through horizontal threaded rods and posts. This stepwise connection pulls one ring relative to the other along the tangent of the rings. In other words, with three or four of these tangential horizontal stepwise connections, the rings will rotate one to the other in a controlled fashion. If all of these connections are placed parallel to each other such that there are three or four step connections between the rings with horizontal threaded rods, all oriented in one

direction, then gradual movement along these threaded rods will translate one ring to the next. In this way bone segments can be rotated or translated one to the other. More complex configurations can even allow combinations of angulation, rotation and translation simultaneously. The key to deformity correction is preoperative planning. A more detailed discussion of deformity corrections and the methods used is described in a recent publication.[39]

While bony deformity correction usually involves correction through an osteotomy or a nonunion, deformities may also be corrected through joints. For example, when there is a joint contracture, distraction of the concave aspect of the contracture will lead to elimination of the soft tissue contracture (Figure 13). For example, in a knee flexion contracture, the antefulcrum rings are placed at the distal tibia and in the proximal femur. The fulcrum wires are placed on half rings on opposite sides of the knee joint. Distraction between the posterior aspect of the proximal femur and distal tibial rings or compression between the anterior aspect of the distal femur and proximal tibial half rings leads to gradual distraction of the concave aspect with stretching and lengthening of all of the soft tissues on the posterior aspect of the knee. This allows a safe correction of severe knee joint contractures without risk of neurovascular damage. Flexion contractures of the fingers may be treated in the same manner. In the foot, severe deformities can be eliminated without the need for osteotomies. For example, untreated or relapsed club foot deformities in older children and adults can be fully corrected by simply untwisting the foot slowly with the wires of the apparatus. It is still unclear as to what is happening to these bones and joints. It seems that by the end of the correction the bones frequently have changed in shape, as have the joints. Distraction across bones and joints leads to vascular changes, bony changes, and joint changes, even in the adult, leading to transformation of their shape.[40] It is not inconceivable that the same principle could be implemented in craniofacial surgery to change the shape and growth direction of the various bones of the face and skull.

In addition to the treatment of the above mentioned deformities, deformities of limb contour can also be corrected. Ilizarov has developed a method for the widening of the calf for the treatment of the thin, asthenic calf which results post polio, club foot, or of congenital origin.[41] This involves pulling bones or segments of bones laterally so as to drape the soft tissues over these bone segments and shape the limb around them.

67

In the leg this is performed by splitting the tibia sagittally into two fragments and then distracting these two fragments in a V shape (Figure 14). This creates a contour on the medial aspect of the leg which resembles the normal bulge of the gastrocnemius seen medially. The apex of the V should be at the level of the maximum medial bulge. On the lateral side the same is performed with the fibula. It is not necessary to split the fibula but rather to divide it at two locations so that it too assumes a V shape. This creates a contour on the posterolateral side which should have its apex at the level of the maximum lateral bulge of the normal leg. On the side of the tibial split, bone regenerates from the medullary canal laterally to fill in the gap. The trabeculae of this new bone are transverse and not longitudinal in their orientation (Figure 14). This demonstrates that trabeculae follow the direction of distraction rather than the orientation of the limb segment. On the fibular side, one sees bone regeneration filling in the concavity of the fibula due to the pull on the interosseous membrane. Ilizarov points out that the change in vascularity created by these tension stresses contributes and controls these form-shaping processes. Due to the large surface area of regeneration of the tibial split, this method has been used by Ilizarov to regenerate a low resistance vascular runoff bed which can be used to treat peripheral vascular disease. This lateral pull technique may have potential application in craniofacial surgery where contour defects are not an uncommon problem.

Other cosmetic applications of the Ilizarov technique include those of lengthening patients for stature.[43] While many patients obtain a significant functional improvement due to their ability to access various facilities in society and even improve their job marketability, there is a significant improvement in their physical appearance. This is performed for conditions such as achondroplasia (Figure 15), hypochondroplasia and constitutional short stature. This technique allows us to change the stature of an individual up to 12 inches by lengthening both tibiae and both femora in two sessions of treatment and the arm in one session by lengthening both humeri.

NONUNIONS, BONE AND SOFT TISSUE DEFECTS
AND OSTEOMYELITIS

The treatment of nonunions depends upon the type of the nonunion. Conventionally, we are used to considering nonunions as hypertrophic or atrophic and infected and

noninfected. Ilizarov's approach to nonunions is much more complex and individualized. He considers a wide variety of factors on each nonunion and based on this decides the most appropriate treatment. One of the most important of these parameters is the stiffness of the nonunion. The stiffness of the nonunion gives information on the tissue between the bone ends. A very stiff nonunion implies that the tissue is either dense, fibrous or fibrocartilaginous tissue, while a lax nonunion implies that the tissue between the bone ends is either loose connective, or synovial. In the stiff nonunion the potential for bone regeneration from the bone ends is present. The dense fibrous or fibrocartilaginous tissue acts like an interzone and when put under distraction regenerates trabeculae of new bone from the nonunion site. On the other hand, in the lax nonunion, little or no bone regeneration results upon distraction. Therefore, bone shortening with a stiff nonunion can be treated by lengthening through the nonunion site itself while the lax nonunion requires compression of the bone ends to convert it to a more stiff type and to lead to eventual union or possibly open reduction and freshening of the bone ends and opening of the medullary canal to allow revascularization of the region and bone healing.

One of Ilizarov's concepts is that of bone loss. Most of us think of bone loss as a bone defect. Ilizarov emphasizes that bone shortening is another type of bone loss. If there is 6 cm loss of bone, it may be manifested as a 6 cm bone defect, a 6 cm shortening of the limb, or a combination of bone defect and shortening of 6 cm. The treatment for each of these situations is different but similar.[44]

The Ilizarov treatment for a bone defect is a technique called bone transport.[45] In this technique an intercalary segment is created at one end of the defect by a corticotomy of the bone. The intercalary segment is then transported at 1 mm per day across the bone defect. Just as in a leg lengthening, bone regeneration occurs between the bone ends. When the migrating fragment reaches the opposite end of the bone defect, it makes contact with the bone at the opposite end (Figure 16). Compression of the contact site usually leads to union. Occasionally, a bone graft is required to expedite this union. The original bone defect has been replaced by a distraction gap at a different level. The length of the distraction gap is the same as that of the original bone defect. This new bone formation undergoes the same stages as that of limb lengthening. When complete consolidation of both the nonunion site and the distraction osteogenesis site is complete, the apparatus can then be removed. In a bone transport the migrating segment is

moving within the soft tissue sleeve. An exact understanding of what is happening at the interface between the transported bone and its surrounding soft tissues is not understood.

In order to transport the intercalary segment, a variety of different techniques may be used.[45] The transport wires may be transverse in which case they need to cut through the soft tissues of the limb in order to advance with the moving bone segment. Alternatively, one may use oblique olive wires to perform the transport, in which case there is less cutting of the soft tissues by the wires. Unfortunately, this is a less stable configuration and more difficult to manage. It is a preferrable method to use in long transports. The transverse wire method is preferrable in short transport distances of less than 5 cm. Finally, I have started inserting wires into the intercalary segment through its medullary canal to the canal on the other end of the transport defect. With an intermedullary guide and transport wire in place, there is no cutting of the soft tissues by the transport wires and there is no missed contact with the transport bone segment passing the opposite bone end like two trains in the night. It insures that the two medullary canals will come opposed to one another.

The technique of bone transport is very useful in the treatment of osteomyelitis. The infected necrotic bone is resected and then treated by bone transport. If there is an associated soft tissue defect, this does not pose any greater problem. In addition to transporting bone, one can transport skin and soft tissues together with the bone (Figure 18). Thus, one can leave the infected wounds of osteomyelitis open to drain as it will gradually close from the inside as the bone transport carries the soft tissues with it across the gap. This precludes the need for many muscle pedicle or free flaps for the coverage of soft tissue defects. There is a lot of potential for the soft tissue transport technique both on its own and in association with bone transport.

The results reported by Ilizarov's coworkers for bone transport in the treatment of 170 tibial bone defects of which 64% had chronic osteomyelitis are: 100% union with elminination of deformity in all cases, limb length discrepancy (LLD) in all but 28.7% of cases, and infection in all but 2.4% of cases.[46] The results in 154 femoral bone defects ranging up to 23 cm of bone loss were union in 147 and elmination of infection in 41 of 45 cases.[47]

In a personally and independently conducted external review of 25 consecutive cases with six month to five year followup treated in Italy, Paley *et al.* reported the

following results: Union in all cases, LLD elminated in all but one case, deformity in all but 4 cases, and infection in all but 3 of 13 with osteomyelitis.[48] The average treatment time was 13.6 months. The average bone loss was 6 cm, ranging up to 23 cm.

How do these results compare to those of conventional techniques and in particular those of free fibular transfer? The most comparable series is that of Goldstrohm *et al.*[49] They reported 6 peroneal nerve palsies in 39 patients, some of which required 38 staged surgeries. Limb length was fully corrected in only 61% of lengthenings greater than 5 cm.

Weiland *et al.* reported an 87.5% success rate of free fibular transfers for noninfected bony defects.[50] Full weight bearing was delayed until the graft hypertrophied (average 15 months). Four failures went on to amputation. In 33 infected bone defects, 5 five went on to amputaiton and 5 of 27 successful transfers had persistent drainage. Only 12 patients walked without assistive devices in followup.[51]

The advantage of the Ilizarov technique is that it is relatively noninvasive, has a high success rate and a low complication rate, while allowing immediate early weight bearing. It relies on the regenerative capacity of the healthy bone and not the pathologic region. Finally, it is a comprehensive treatment which addresses not only the problem of the nonunion and bone defect but also that of the shortening, deformity, infection, and any associated joint contractures.

FRACTURE TREATMENT

Another useful application of the Ilizarov technique is as an external fixator for the treatment of acute fractures. Needless to say it is useful in the management of many long bone fractures, especially in the tibia. It is one of the few fixators that can offer excellent stability, permitting unrestricted weight bearing. Another advantage is in the treatment of metaphyseal fractures with comminution. The treatment time of tibial fractures in the Ilizarov frame has been reported to be lower than that using conventional techniques.[52] The most difficult fractures remain the grade 3 open tibial fractures.[53] The difficulty arises in the grade 3B and C injuries. Grade 3A fractures are those in which there is severe bony and soft tissue damage but where the bone ends can be covered by the deep tissues of the wound, leaving no exposed bone. A grade 3B fracture is one in which bone cannot be covered by the surrounding soft tissues, leaving open,

exposed bone. This latter group has a higher infection rate than grade A fractures. Most recommendations are for early soft tissue coverage using free tissue transfer or muscle pedicle grafts. It is in this latter group too, that the Ilizarov method may offer an alternative solution. The apparatus may be applied and the fracture can be acutely shortened.[26] By shortening the fracture one may convert a 3B to a 3A situation, allowing coverage of the bone with the surrounding deep soft tissues due to the redundancy of tissue from the shortening. This may elminate the need for soft tissue coverage procedures. Subsequent relengthening can then be performed. This acute shortening can only be performed when the fibula is fractured and at the same level of the tibial fracture. In addition, the open wound needs to be transverse or oblique but not longitudinal. If it is longitudinal it will gape open and in fact the situation may be worsened. Therefore, this maneuver is of limited applicability but is it very useful in selected cases. Another useful maneuver is to angulate the fracture towards the open soft tissue, thus gaining in soft tissue coverage on the concavity. The angulation can then be removed at a later date to correct the iatrogenic deformity.

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FIGURE LEGEND

Figure 1

Gavriil Abramovich Ilizarov

Figure 2

Ilizarov apparatus with multi-level multi-planar multi-directional wire fixation.

Figure 3

A human erector set: The apparatus is constructed from modular parts. The dynamometric wire tensioner is shown - No. 33.

Figure 4

A - Distraction Phase: Longitudinally oriented trabeculae form from either side of a central lucent zone - the interzone.

B - Fixation Phase: The distraction complete, the new bone is allowed to mature. Neocorticalization is seen and ossification of the interzone begins.

C - Removal Date: There has been suffficient neocorticalization and obliteration of the interzone to allow safe removal of the fixator and unprotected load bearing.

D - Recanalization: The medullary canal is remodeled and the new cortex is the same thickness as the old. It's almost impossible to tell that a lengthening has occurred.

Figure 5

There is continuity between the nutrient artery and vein and the richly vascular trabeculae of new vessels. The interzone is relatively hypovascular compared to the trabeculated regions which are hypervascular (insert). The interface between the interzone and the tips of trabeculae is shown. Note that the cells surrounding the trabeculae and the cells of the interzone appear similar. It is presumed that the interzone cells are an undifferentiated mesenchymal type cell and transforms directly into

the osteoblasts of the trabeculae. Therefore, the process is one of intramembrànous bone formation.

Figure 6

Schematic of double level tibial lengthening.

Figure 7A

Six year old boy with a 6.5 cm leg length discrepancy.

Figure 7B

Double level lengthening of the tibia to a total of 10 cm in order to compensate for future discrepancy.

Figure 7C

At 6 month followup, the over lengthened tibia is seen.

Figure 8A

Growth arrest of the distal radius with 3 cm of shortening relative to the ulna.

Figure 8B

After 3 cm. of lengthening.

Figure 9

Different types of correction depending on hinge placement.

Figure 10

The "rule of thumb" for fulcrum and antefulcrum placement. The principle is that of four point bending.

Figure 11

A standard mounting of the ilizarov apparatus for an opening wedge angular correction. There are two levels of fixation in each bone segmented, and the rings are perpendicular to the shaft of the tibia. The hinge is placed at the apex of the deformity.

Figure 12A

Valgus deformity of proximal humerus secondary to Ollier's Disease.

Figure 12B

Double level lengthening with hinge placement medial to the apex of the deformity.

Figure 12C

Distraction hinge correction. Note the hinge is now straight.

Figure 12D

11 cm. of lengthening. Removal of apparatus at 17 weeks.

Figure 13A

Congenital contracture of knee joint in 18-month-old girl.

Figure 13B

Application of Ilizarov apparatus with hinge at knee, 2 fulcrums and 2 antefulcrums. Distraction between 2 fulcrums.

Figure 13C

Over correction was achieved.

Figure 14A

Widening of leg. The tibia is split longitudinally and distracted laterally. Note the new trabeculae are in line with the direction of distraction. The fibula is also pulled laterally. Note the new bone in the interosseous membrane. The tibia is being lengthened distally. An interzone is seen between the proximal and distal trabeculae.

Figure 14B

Left: Before correction.
Right: After leg widening, lengthening, and foot deformity correction.

Figure 15A

Standing full length AP radiographs of both lower limbs before and after 16 cm bilateral double level tibial lengthening for stature.

Figure 15B

The patient who has achondroplasia is seen standing next to her mother before the lengthening (left - height 3'11.5") and 8 month later after the lengthening (right - height 4'6").

Figure 16A

6 cm bone defect secondary to trauma, stabilized in Ilizarov apparatus.

Figure 16B

During double level bone transport from above and below. The two transport segments have made contact.

Figure 16C

After removal of the apparatus. Total treatment time one year.

Figure 17A

Bone transport schematic by: longitudinal oblique wires.

Figure 17B

Intramedullary wires.

Figure 17C

Transverse transport wires.

Figure 17D

The final step is compression of the contact site using a ring.

Figure 18A

Radiographs of bone defect at the beginning and end of bone transport. There is a 6 cm. distal tibial bone defect.

Figure 18B

The open wound gradually closed at the transport segment descended. No soft tissue procedure was required. Left - initial wound, center - during transport, right - with the bone transport completed. Note the ring that descended.
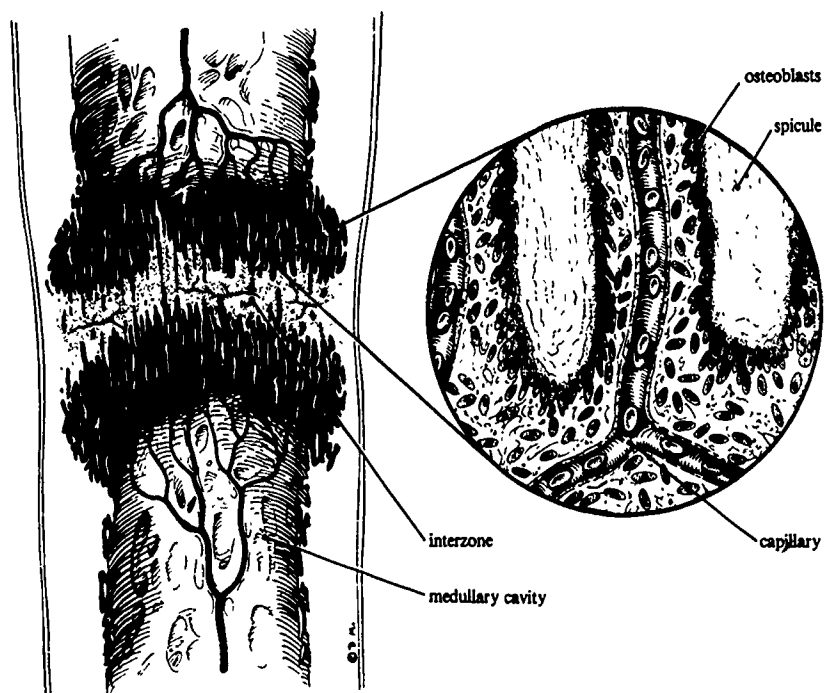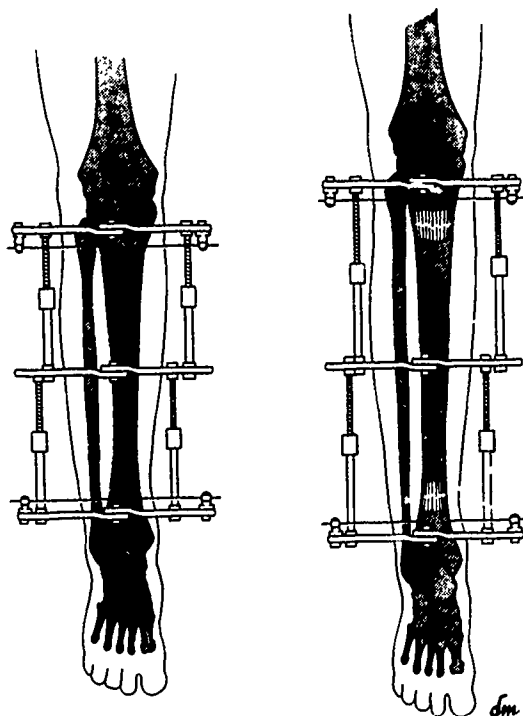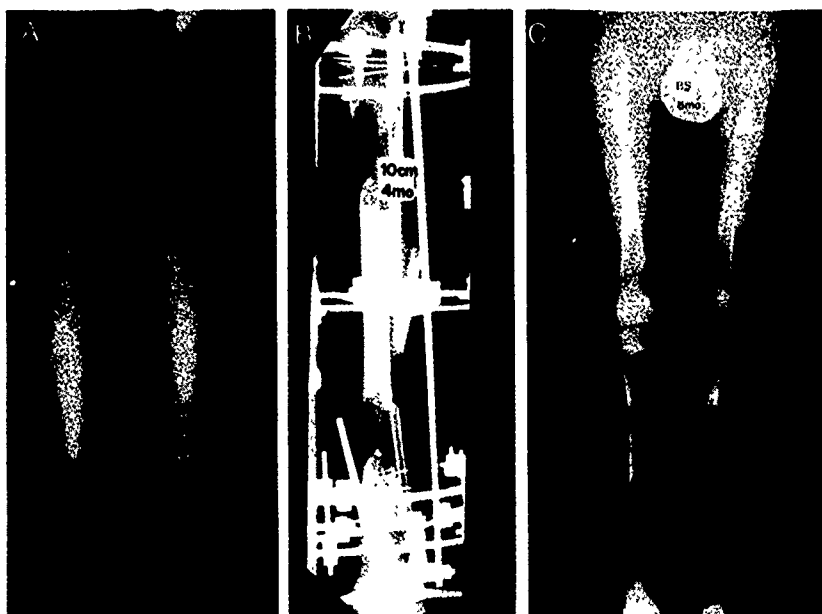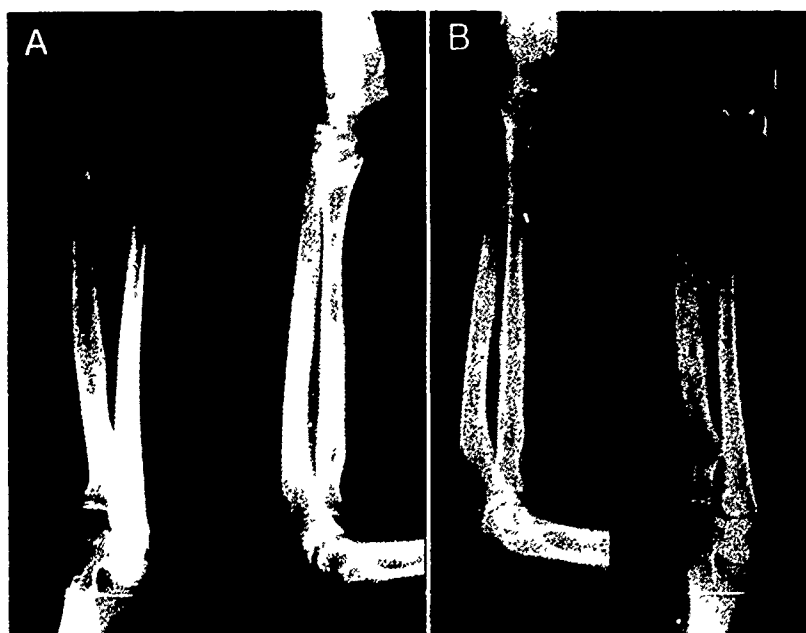
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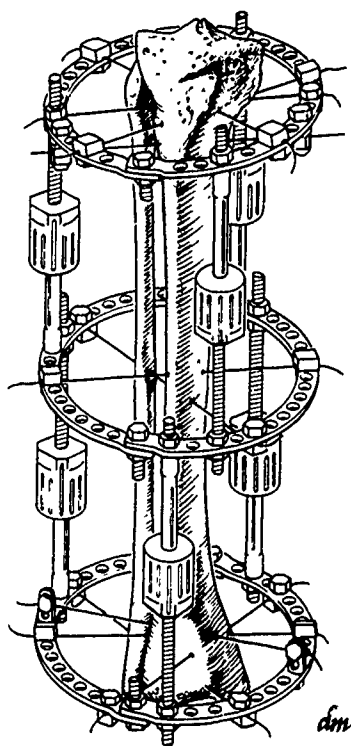
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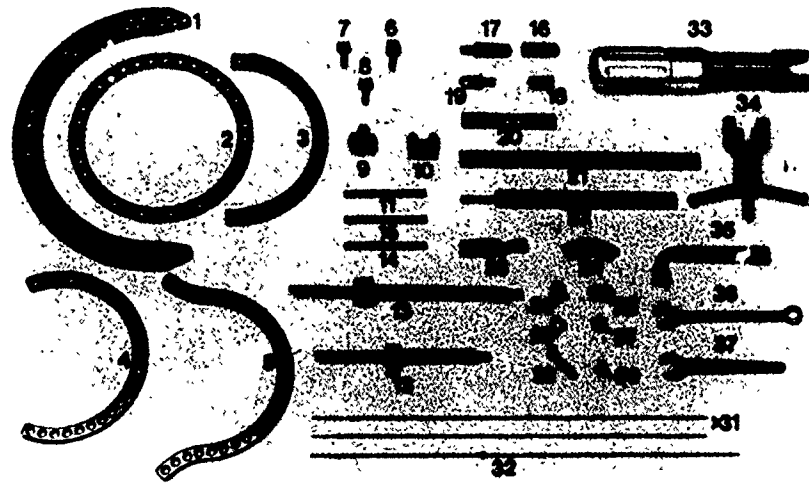
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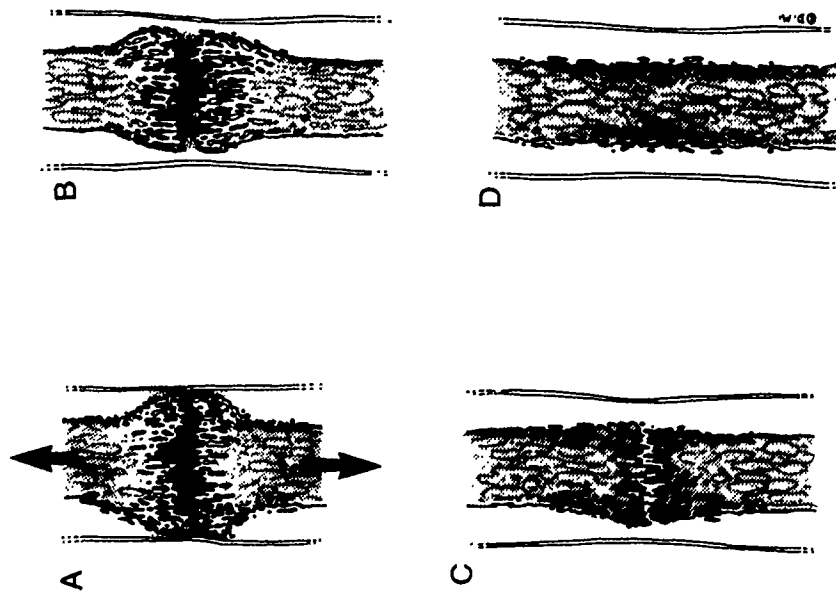
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Figure 5

Figure 6

Figure 7

Figure 8

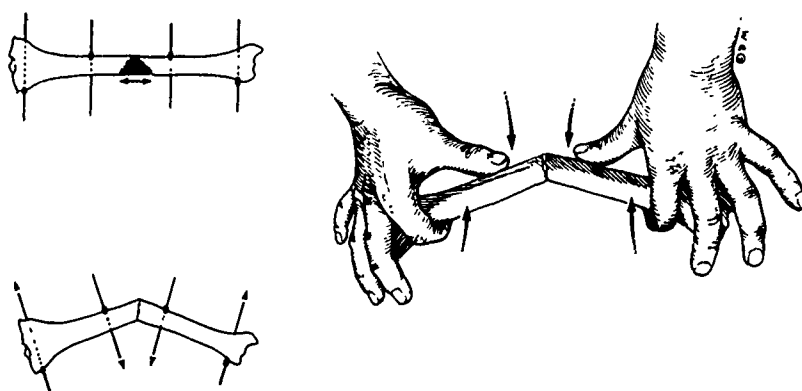OPENING WEDGE HINGE   DISTRACTION HINGE   COMPRESSION HINGE
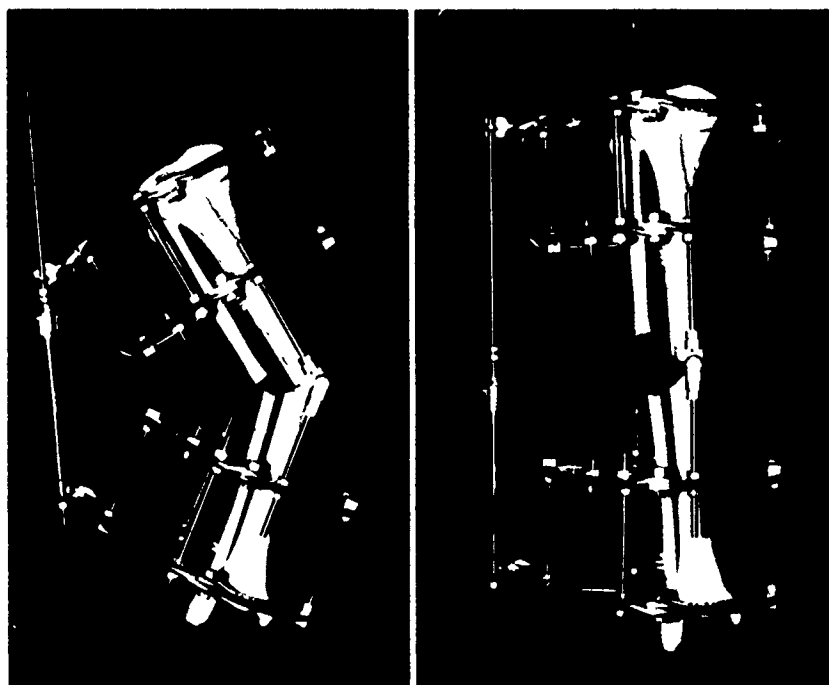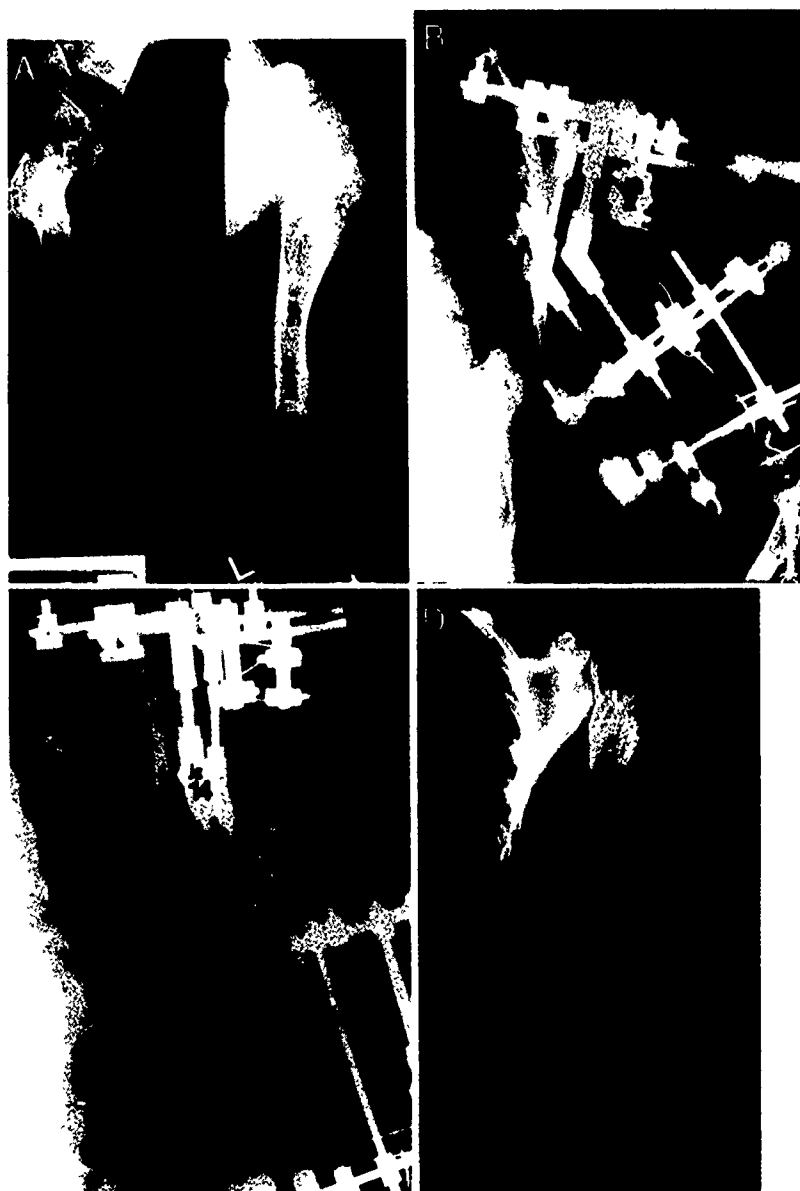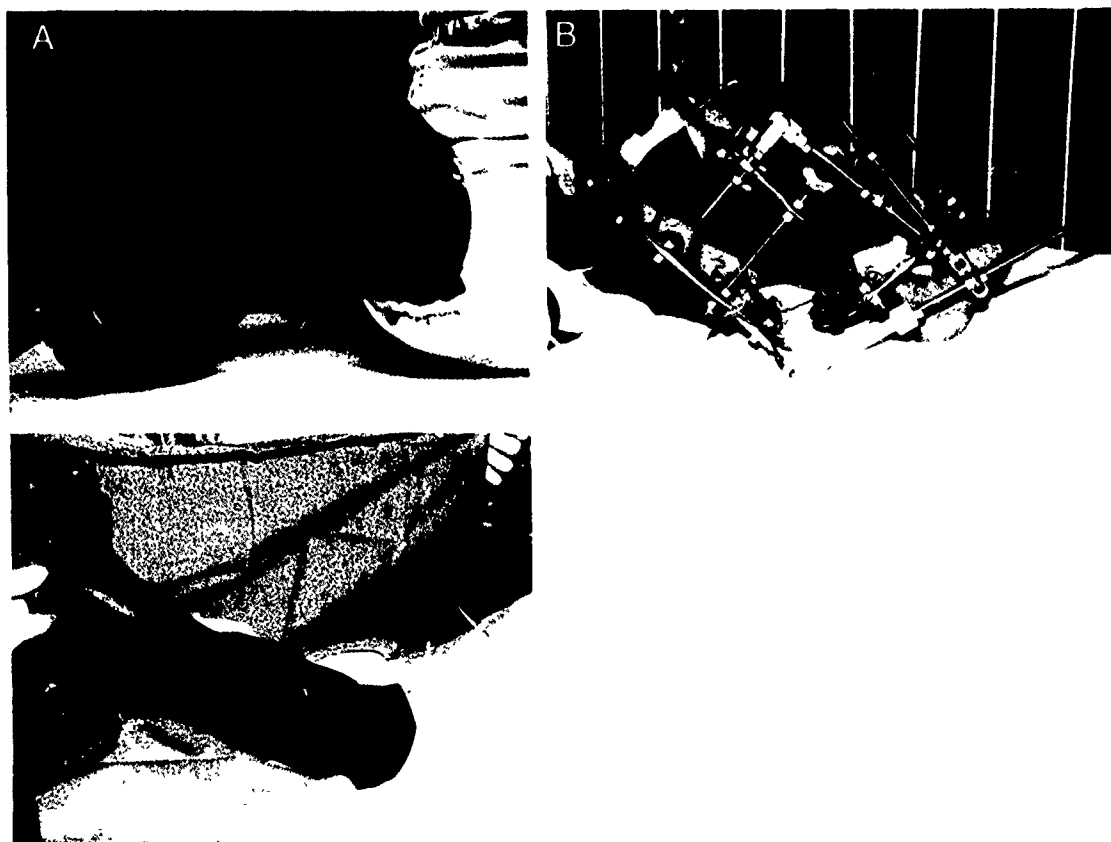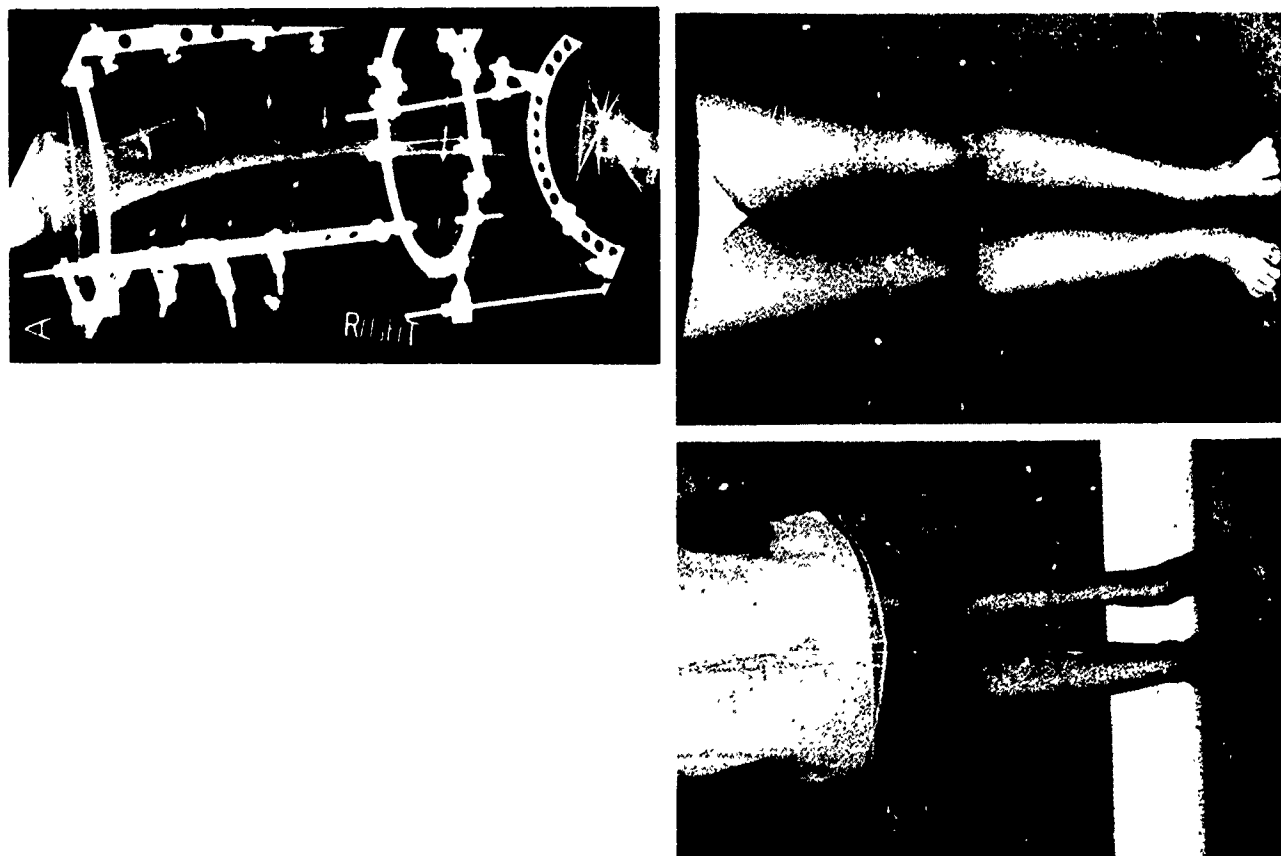
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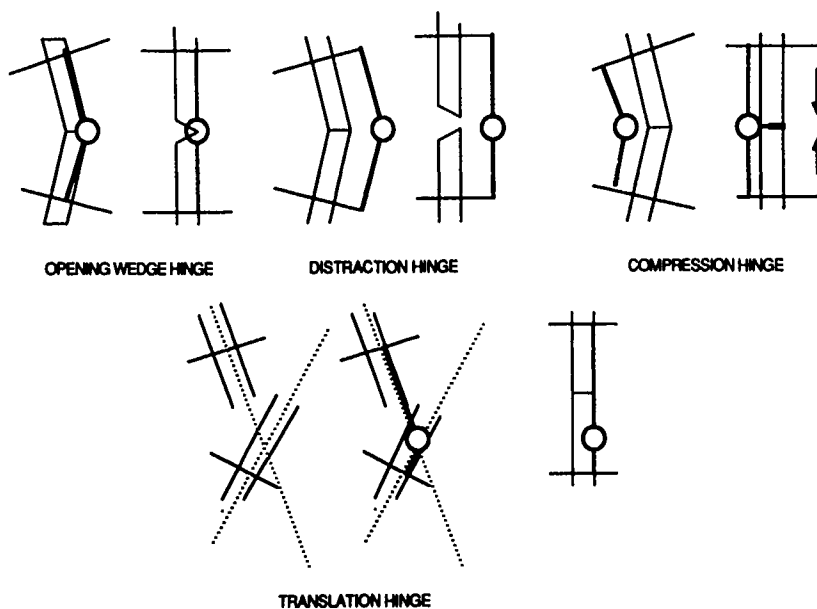
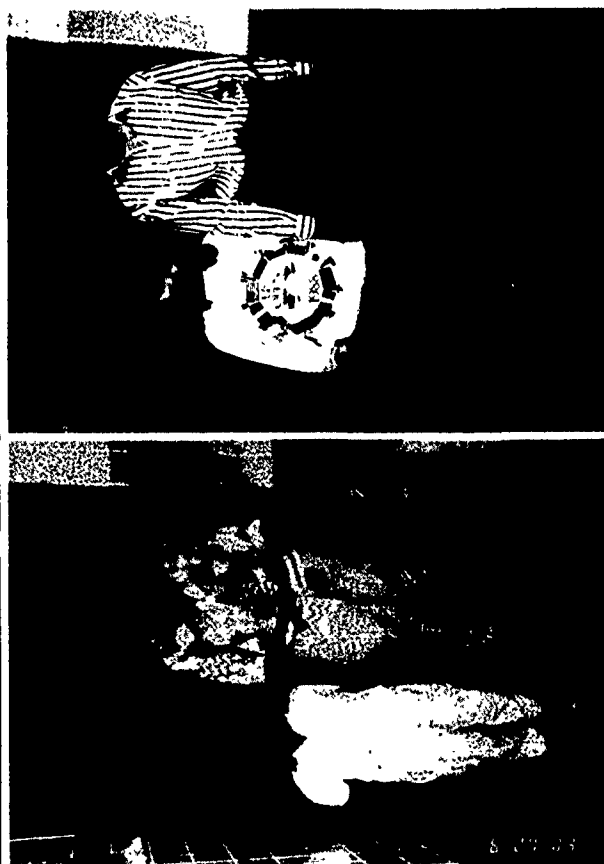Figure 9

Figure 10

Figure 11
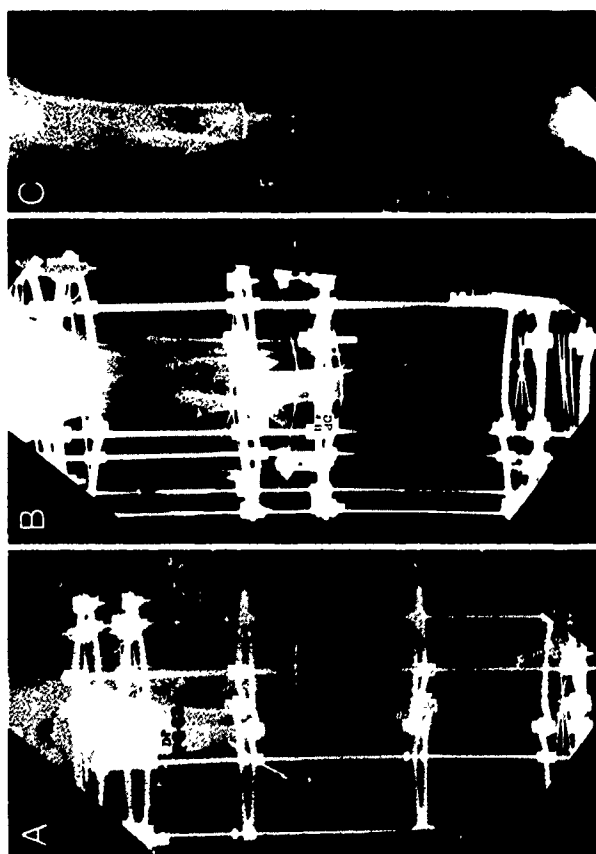
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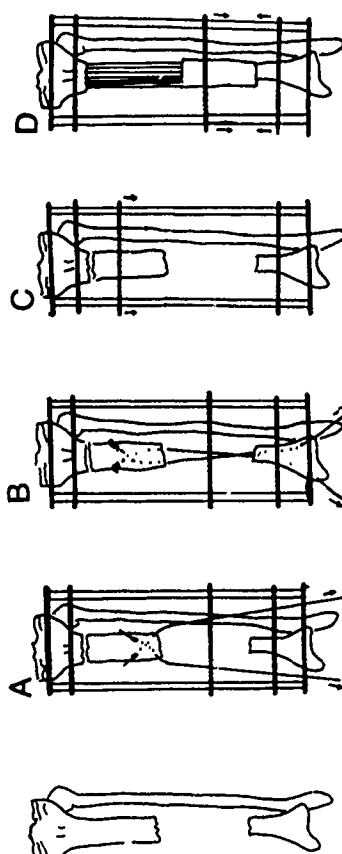Figure 14

Figure 15

Figure 16
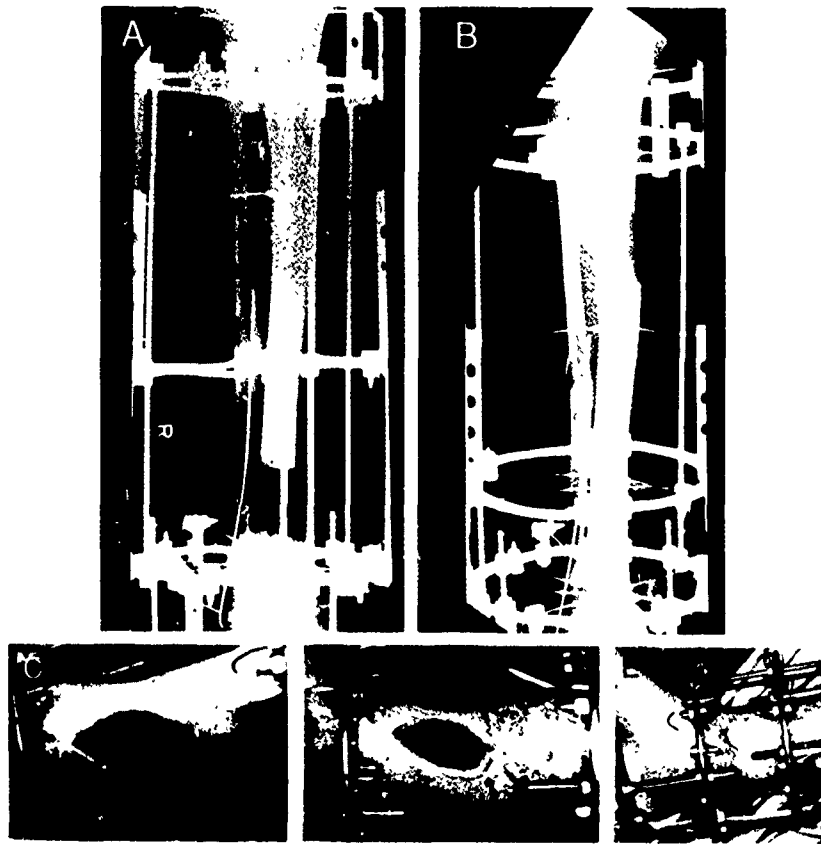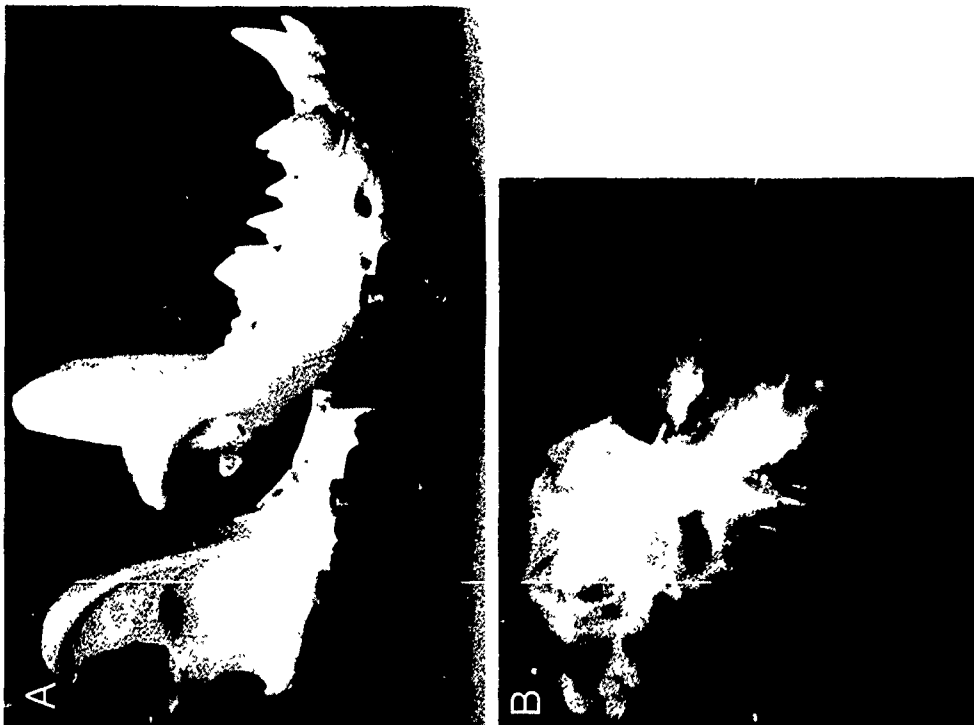

Figure 17

Figure 18

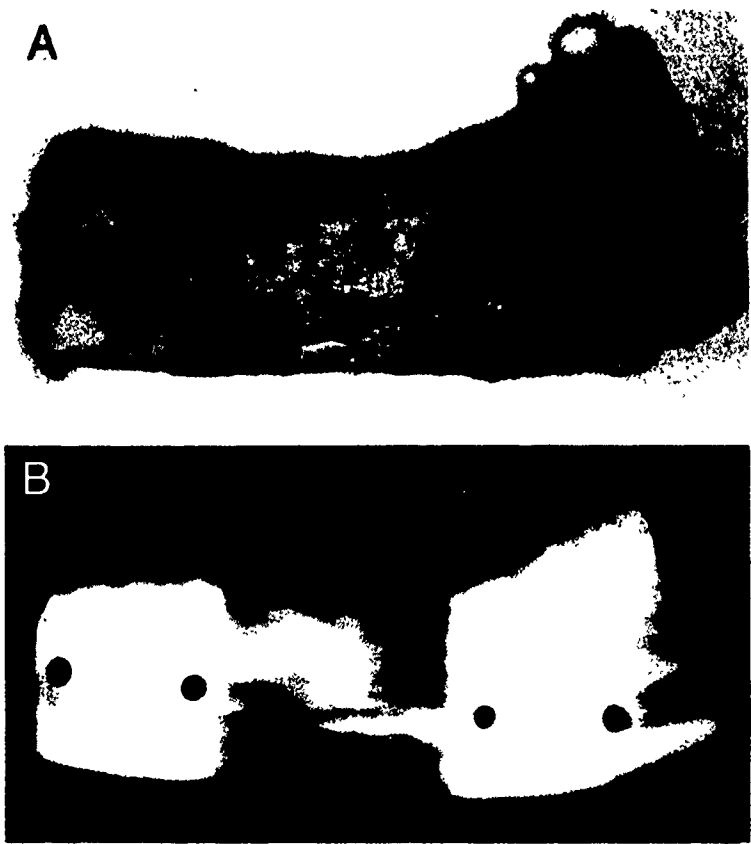Figure 19

Figure 21

Figure 20

Figure 22

Figure 23

Figure 24

BONE GRAFT SYSTEMS IN ORAL AND MAXILLOFACIAL SURGERY

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INTRODUCTION

The reconstruction of osseous defects and deformities following oncologic surgery or trauma, and the restoration of defects of congenital origin, requires the application of appropriate bone graft materials, and proper functional stimulation of the graft post-operatively.

Bone graft materials traditionally have been composed of osseous materials of: 1) autogenous, b) allogeneic, or c) alloplastic origin.

While all of these graft materials have a place in maxillofacial reconstruction, the use of various types has markedly changed with the new application of certain materials, the need for development of new techniques, and the results of research into new bone inductive and non-inductive alloplastic materials.

The primary graft material through the past few decades, however, has remained particulate marrow and cancellous bone (PMCB), usually taken from the iliac crest, although at times taken from other sites (Table 1).

For purposes of discussion, the standard for comparison of grafts is usually iliac crest cancellous bone, because graft material taken from this donor site presents with an optimal mass of hematopoietic marrow, containing more cells capable of forming bone than other donor sites. It is generally accepted that the most effective bone grafting material is fresh autogenous bone, particularly from a marrow-rich site. Except for certain areas such as the iliac crest, bone as a tissue is composed of relatively small numbers of living cells with a large amount of non-cellular intercellular matrix, which may be considered in many cases to be nonviable. Since the portion of bone having the largest number of cells is the marrow-vascular spaces of the spongeosa, a great amount of attention has been given to the study of the transplantation of this type of graft substance.

The mechanism by which these autogenous marrow cells induce osteogenesis in the grafting procedures, has been the subject of a great deal of research investigation during the past decade, and there has been a marked controversy concerning the mechanism by which osteogenesis is induced by such bone grafts or transplants.

Osteogenesis (Osteoinduction, Osteoconduction, and Osteophilic Responses)

Paramount to the understanding of the possibilities of bone regeneration is an understanding of the interpretation of the nomenclature used in the above subtitles, which at times is very confusing. The interpretation of these terms by the research laboratory investigator sometimes is quite different from that of the clinician. We would like to discuss the clinically relevant aspects of the subject terminology.

Bone Induction

To the clinician, the term "bone induction" is synonymous with "osteogenic stimulation". This implies that the bone graft material being used has the ability to induce bone formation in a recipient surgical site which normally would not of itself repair with new bone. Thus, "bone induction" procedures are used primarily to bridge large discontinuity defects or to reconstruct bone on host surfaces where normally there would be no significant repairative response by the host independent of grafting. Bone induction systems are primarily autogenous in nature, particularly when demonstrated by the properties of the autogenous particulate marrow-containing iliac cancellous crest bone, as mentioned above. The cancellous bone matrix containing the marrow spaces has the ability to interact with the cells in the marrow, particularly the pluri-potential prodromal cells of the marrow itself (Fig. 1).

In reviewing the cell types contained in the marrow vascular spaces of particulate cancellous bone, we may find that there is present a milieu of cells; any number of which could be responsible for the formation of bone. It is now generally accepted that it is the cells of the syncytium net, and the vascular lining of sinusoids which are pluri-potential. These cells have the ability to mature and differentiate in a number of ways. The induction of these cells to form along osteogenic lines and to form bone is the basis of the clinical success of autogenous particulate grafts.

The hc st cell bed also contains the same types of cells, but to a lesser degree. So there are these two possibilities of osteoinduction in the stimulation of pluripotential cells. The stimulation of the graft itself by autoinducing its own cells to form bone producing elements and the stimulative effect of the graft on the host cells to do the same thing (Fig. 1).

The pluripotential target cells of the marrow-vascular spaces are morphologically very innocuous and are not remarkable histologically, but they are capable of developing in a number of different directions or tracts. For example, they can become fibroblasts or fat cells or osteoblasts, or they can become cells of the hematopoietic series.

Within the marrow vascular spaces of the graft, there are also surviving osteoblasts and pre-osteoblasts or cells which have already been committed to becoming bone forming cells in the graft. Such bone formation from existing osteoblasts and committed osteoblasts is an initial part of the "success" of the particulate autogenous graft. This is a very minor part, however, of the success of the graft, and the effect is very short lived. It tends to be emphasized by some investigators as a so-called "Phase I" as against "Phase II" of post-graft bone formation, with Phase II being true induction. In reality, the visible formation of bone radiographically and histologically (Fig. 2) in most graft systems is, for the most part, the result of bone induction or the so-called Phase II. The dwelling on a "Phase I" healing component can only detract from the true problem, and the major possibility of bone formation from grafting. The clinical success of a graft is in the inductive phenomenon, rather than through surviving osteoblasts.

In summary then, bone induction can come from the graft material inducing itself (its own pluripotential cells) and from the graft material inducing the primitive cells of the recipient host bed.

Bone induction involves phenotypic changes in pluripotential cells to form a desired bone-forming cell line. It is possible that this phenotypic change can involve the formation of cartilage. In certain laboratory experiments, this is quite evident; but clinically, the formation of the cartilage following bone-inductive surgical procedures (i.e., grafting) usually signals that there is either a decrease in blood supply to the area or movement of the bone fragments producing these changes. True bone induction need not go through a cartilaginous phase, and in fact, to the clinician, the cartilaginous phase in facial bone reconstruction is to be avoided.

Experimental studies producing cartilaginous bone formation in ec. '.' soft tissue areas, are not, of themselves, particularly relevant to the clinician.

Bone Conduction

Bone conduction as opposed to bone induction simply implies that any cellular system has the ability to influence other cells which have already been programmed to become osteoblasts to differentiate more efficiently and more expeditiously in producing bone formation. Bone conduction takes places in already predetermined or programmed cells, and not in the pluripotential primitive cells that have the ability to differentiate in many different areas, but have not yet made that cellular "decision" and are awaiting the bone inductive stimulus from other sources.

Clinically, we can best demonstrate bone conduction by observing the placement of graft upon a host bone surface and the apparent effect of the graft in leading repair from that surface. The effect is usually short-lived and would not maintain itself unless accompanied by added bone inductive stimuli.

The bone conduction occurring in intraosseous defects, however, can lead to a complete regeneration of the defect in a more expeditious fashion, than if the intraosseous defect was allowed to regenerate by the simple process of bone forming from the peripheral surfaces of the deficient area. A good example of the latter is a solitary cystic defect of the bone of the jaws, which can be aided in a more rapid osseous regeneration by osteoconductive-type grafts, such as freeze-dried cancellous banked bone.

Osteophilic Response

Osteophilic reactions simply imply that the bone "graft" material produces a surface upon which the host bone can grow, and which can be the site of some limited amount of bone formation by a surface or nidus effect. There are many synthetic alloplastic materials which possess osteophilic properties. The basis for this response lies only with the acceptability of the surface material to cellula. proliferation, whether the cellular proliferation is osteoblastic, fibroblastic, or endothelial or epithelial. A good example of osteophilic response is the formation of bone along the surface of a Millipore, or Gortex filter. If the filter has an appropriate pore size, cells are excluded from migrating through the surface of the filter to the opposite side, but can migrate along the

epsilateral filter surface, producing the effect of a limiting boundary with bone forming on one surface and fibrous tissue on the opposite side. Such growth can be used to exclude fibrous tissue form a regenerating defect of the jaws or face, giving the normal osseous repair response the opportunity to regenerate the defect independent of any graft material. Use of guided tissue regeneration by filter membranes was first reported in 1961 by Boyne. A Millipore filter* was used to exclude fibrous tissue in bone grafting of large bony defects.[1-3] Osteophilic responses are sometimes confused with osteoconduction. A good example of this is the use of nonporous particles of hydroxyapatite in the augmentation of the mandible. The particles lying next to the host bone surface may be bonded to the bone, but the effect of the particles in stimulating either conduction or induction, is negligible or completely nonexistent. More pheripherally placed hydroxyapatite partic.es in mandibular and maxillary bone augmentation procedures are merely surrounded by fibrous connective tissue.

The bonding, therefore, of such materials to bone is an phenomenon which can be used in certain types of reconstruction, such as the use of intraosseous metal implants coated with hydroxyapatite, to facilitate bonding to bone, but the induction or conduction of new bone by these alloplastic materials in particulate or large block form is usually nonexistent.

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REMODELING AND
FINAL RESULTS OF BONE GRAFING PROCEDURES

</div>

It is not enough to produce an initial induction of bone formation in the surgical repair of facial bone areas, but the graft material must have the ability to remodel and reconstruct itself to a type of osseous tissue which will resist resorption over a long period of time and to resist atrophic changes. It is well known, for example, that the atrophic alveolar ridges of the jaws may be grafted to an optimal height and width by autogenous bone grating procedures, but the _maintenance_ of this height and width is a significant problem in corrective bone reconstruction. Usually autogenous bone grafts when used in this manner result in remodeled bone which has a very fine trabecular pattern, which is very prone to resportion when subjected to the demands for

*Millipore Corp., Bedford, MA

mineral release through homeostatic changes occurring systemically in the host patient, and through the local effect of prosthesis or other stresses being brought to bear on the grafted surface.

One of the methods that has been employed with some degree of success during the past few years, to effect optimal remodeling of the grafted mass in maxillofacial defects, has been the incorporation within the autogenous particulate marrow and cancellous bone of particles of inorganic material, such as porous hydroxyapatite** or xenogeneic animal bone mineral***. The effects of these particles with the osteoinductive marrow vascular cells of the PMCB graft has been to produce a final remodeled surface which has a heavy trabecular pattern and a thickened lamellated bone surface. This regenerated area with increased lamellated bone and increased trabecular bone density tends to resist resorption. Animal and clinical studies in which porous hydroxyapatite and porous bone material with autogenous bone has been used to reconstruct edentulous alveolar ridges, has lead to retention of the grafted area and a relative lack of resorption by the reconstructive production of bone surfaces which are long-lasting and serviceable to the patient in terms of prosthetic function.[4]

THE EFFECT OF POST-GRAFT OTHOPEDIC STIMULI ON THE OVERALL SUCCESS OF THE GRAFT

Perhaps more important than the type of graft material and the final remodeling end-result of the autogenous graft is the type of functional stimuli which are applied to the graft after it is placed, both on a short-term and relatively long-term basis. The graft can either fail or be successful, depending on the types of pressure and/or tension and other stimuli that are brought to bear on the cellular substrates of the grafted mass. In orthopedic surgery, a constant concern is the development of methods of bringing about appropriate stresses and proper forces on grafts placed in weight-bearing bones. In the reconstruction of the jaws, there is a readily available method of stimulation of bone grafts, i.e., functional prosthodontic construction and appropriate orthodontic appliance application. This can be demonstrated by placement of graft materials in various defects of the jaws, particularly cleft palate defects.

** Interpore 200, Interpore International, Irvine, CA

***Bio-Oss, Geistlich and Sons, Wolhusen, Switzerland

CLEFT PALATES

A recent review was made of cleft palate cases operated on our Service between 1970 and 1991. In this period of 21 years, over 450 cases were operated. 268 were available for evaluation and 70 cases were complete in their evaluation documentation and follow up. The follow up period was between 6 and 20 years. In reviewing these unilateral and bilateral cases, it was found that orthodontic expansion after PMCB iliac crest grafting is preferable to expanding before placing the graft. The effect of orthodontic stimulation between 3 - 6 months after placement of graft material is one of inducing the graft to form calcified matrix and actually enlarging the bone mass.

Such orthodontic stimulation may be by movement of the central incisor teeth or by the actual eruption of teeth through the grafted area (e.g., canine eruption) or by expansion of the arch transversely in the area of the graft (Figs. 3 and 4). Any of these orthopedic forces of orthodontic movement can stimulate the graft not only to be successful but to actually remodel and tend to duplicate the normal maxilla. There has been no disconcernable detrimental effect on the osseous growth of the maxillary arch or the maxilla itself by such grafting. Arch form in the large group of patients was excellent.[5] Semb[6] reported on the similar group of over 500 patients that the crossbite of the incisor teeth could be corrected at the age of 7 and that grafting prior to the canine eruption would stimulate the graft to mature. It has also been noted that if the use of othopedic forces of orthodontic treatment is delayed for 2 - 3 years after grafting, then various complications occur such as tooth impaction, narrowing of the arch, difficulty in arch expansion and a tendency toward relapse of the arch once it is expanded.[5]

We have, therefore, recommended that orthodontic stimulation of the graft be instituted within a period of 3 - 4 months (Figs. 3 and 4) after grafting when a cross bite malocclusion or malpositioning of the incisor teeth is evident.[5]

Thus, the appropriate stimulation of the graft in the host bed is extremely important to the overall success of maxillofacial resotration.

RAMUS LENGTHENING

The lengthening of the ramus by bone grafting followed by various types of binator and Frankle appliances which place the mandible in a functional position and tend to distract the condyle from the glenoid fossa after ramus grafting have been shown to stimulate bone grafts. Such reconstruction of the temporomandibular articulation and

postoperative stimulation produces a satisfactory ramus in cases of hemifacial microsomia. Thus, the stimulation of the growth of the graft in the microsomia patient is extremely important.

CONCLUSION

1. The base-line comparison graft in maxillofacial reconstruction is still iliac crest particulate marrow and cancellous bone, although other autogenous grafts are effective in various areas of the facial skeleton.

2. The use of bone inductor materials and improved alloplastic materials for use in the esthetic reconstruction of the face certainly will develop more sophistication in the future.

3. The use of various porous alloplastic materials such as porous hydroxylapatite with autogenous PMCB in any composite graft has been very effective in producing a final remodeling which is resistant to resorption.

4. Remodeling of the graft to produce optimal reconstructive characteristics of the restored area is as important as initial induction of bone formation.

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FIGURE LEGEND

Figure 1

A chart depicting the effect of a particulate marrow cancellous bone graft on its own pluripotential cells to produce bone formation. The lesser activity of surviving osteoblasts is also shown. The effect of the autograft on the pluripotential cells of the host bone defect is also shown. Both of these tracts involve pluripotential cells with the main thrust of the graft material on its own cells and the pluripotential cells of host leading to the full expression of bone induction.

Figure 2

A decalcified specimen taken two weeks after graft placement showing a proliferation of osteoblasts between two areas of calcified bone matrix. The osteoblasts are a result of the induction of the pluri-potential cells of the marrow-vascular space of the grat that is shown, and not mere surviving of osteoblasts which were juxtaposed to the original bone graft matrix surfaces ·· the time of surgical placement

Figure 3

A clinical view of the effect of orthodontic movement of a canine tooth on a grafted cleft palate. The bone has been grafted with PMCB from the iliac crest 3 months earlier. Note that the bone at the crest of the ridge "following" the occlusal movement of the tooth. The effect of tooth movement and eruption is an optimal one producing differentiation of osteoblasts from pluripotential cells and formation of bone after grafting.

Figure 4

An arch completed by orthodontic expansion on the movement of the teeth after grafting of a bilateral cleft palate. The alveolar ridge has obtained optimal height and there is excellent bone formation around the canine teeth which have been brought to the central incisors in the area of the cleft bilaterally. Such tooth movement through the clefts has maximized the bone graft, produced normal bone structure, and the bony ridge is intact for long-range periodontal and alveolar ridge bone maintenance.
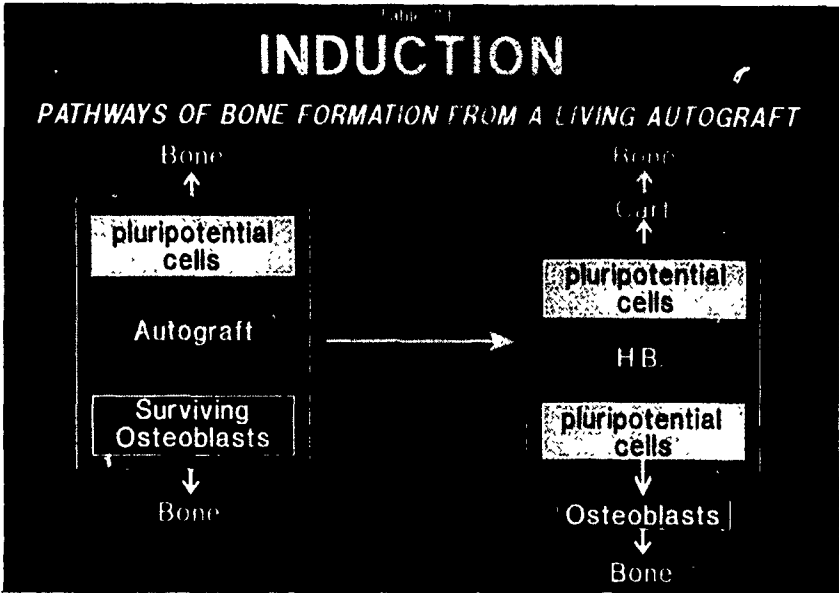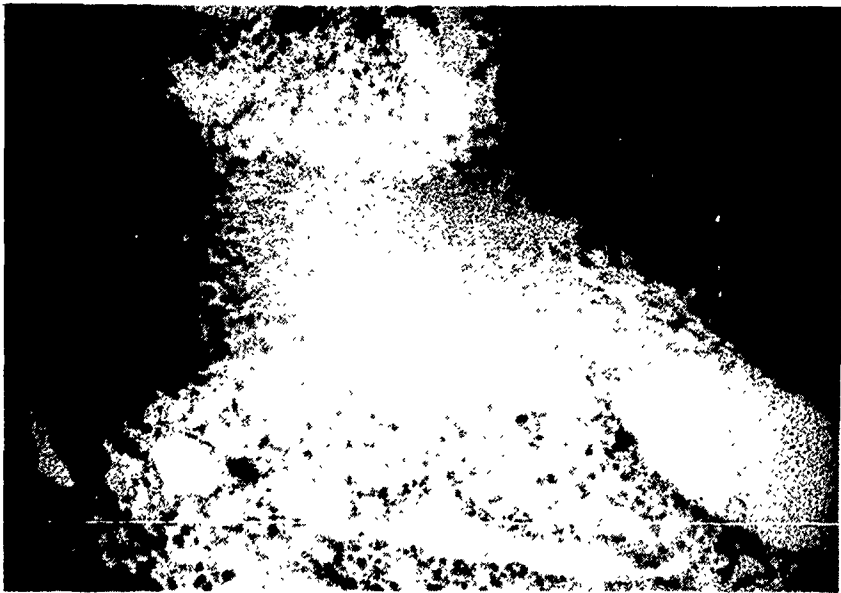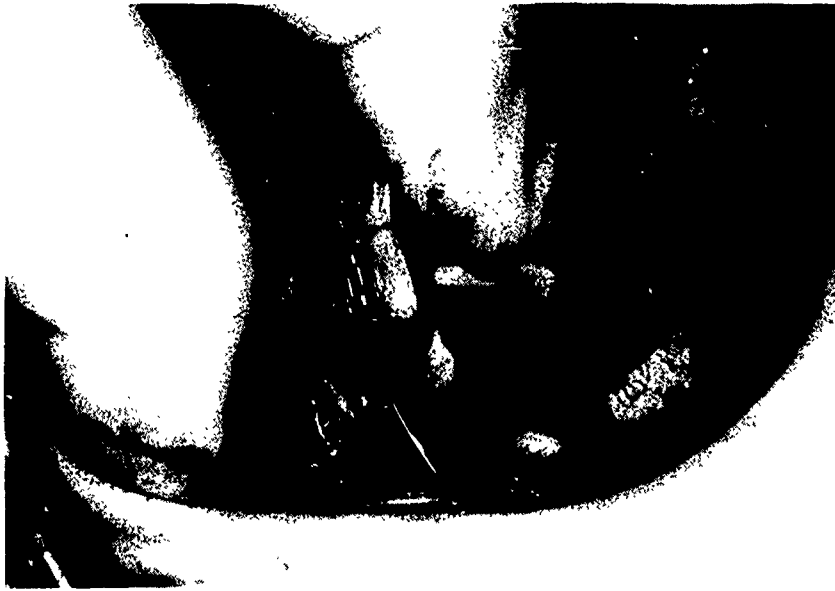
Figure 1

Figure 2

Figure 3

Figure 4

TOWARD THE 21st CENTURY
CT BASED FACIAL FRACTURE TREATMENT

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CT FRACTURE TREATMENT

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INTRODUCTION

Facial fractures have formerly been classified by the anatomic region of the face in which the fracture was located. We speak of fractures of the upper and lower jaw, the zygoma, orbit, nose and frontal bone. The degree of comminution within a particular anatomical area may be quite different however, and the treatment required varies considerably with the pattern of the fragmentation within a specific anatomical area.

In the days of plain radiographs and closed treatment of facial fractures, the designation of anatomical area alone provided sufficient classification. With the increasing application of extended open reduction throughout an area of fracture, a more extensive knowledge of the pattern of the fracture has been required to guide exposure and fixation. This information has been provided in abundance by the use of computed tomography. This CT derived information, supplemented by stability and result data gained from experience with open reductions which utilize craniofacial exposure and rigid fixation to reconstruct the buttresses (Figure l) of the facial skeleton, may be combined to produce recommendations regarding exposure and fixation for various degrees of comminution observed within a specific anatomic location.

ANATOMICAL CONCEPTS

Several authors have previously provided classifications of fractures within anatomical areas in an attempt to clarify treatment options within that region or to provide a basis for comparison of results. LeFort,[1] in a series of cadaver experiments, identified the usual patterns of comminution and the areas where segmentation occurred in LeFort fractures. His results were simplified to indicate only the upper level in each case where mobile midface skeletal segments were separated from stable facial structures. This simplified by Adams[2] "LeFort Midface Fracture Classification" had practical significance in that it identified the level above which suspension wires had to be directed in order to achieve stability by treatment consisting of impaction, suspension wires and intermaxillary fixation.

Stranc,[3] in evaluating fractures of the nose, classified these injuries into "laterally deviated" fractures and "frontal impact" injuries. The frontal impact injuries were subclassified into planes 1, 2, and 3, according to increasing involvement of the supporting bones of the nose, that is the frontal processes of the maxilla. Plane 3 injuries actually extended outside the true nasal skeleton to involve the nasoethmoidal-orbital region.

Sturla[4] evaluated the angle of impact, patterns and mechanisms of craniofacial fractures in cadavers. He identified central and lateral injury patterns (Figures 2 and 3), and documented their patterns of extension and the effects of energy on segmentation of the upper and mid facial skeleton. Stanley[5] indicated that, for LeFort fractures, the fracture pattern corresponds to the direction of force creating the fracture. Lateral impacts produced a different pattern than frontal impacts. Sofferman,[6] Stanley[5,7] and Rowe[8] have each commented on the patterns of maxillary fractures and emphasized the need for vertical stabilization. Manson[9-11] and Gruss and McKinnon[12] identified common patterns of LeFort fractures and recommended classifications which identifies individual fractures present within each LeFort level. These schemes and others[13-16] identifies the areas where stabilization is required in the buttresses of the maxilla and zygoma. Gruss and McKinnon[12] also emphasized the buttress system in stabilization of the maxilla.

Gruss classified nasoethmoidal-orbital fractures by describing the patterns of extension, relating the patterns of fracture to the exposure required for each injury.

Hagan[19] has described mandibular fracture patterns. Manson[20] has also characterized the patterns of fractures in the frontal bone, nasoethmoidal orbital area, and zygoma[21] (as has Jackson[22]) and emphasized a buttress scheme for reconstruction.

A revolution in facial injury treatment has occurred in the last 10 years following the application of craniofacial techniques of exposure and fixation to the full open reduction of the facial skeletal injuries. Operations are dramatically different than those first suggested by Milton Adams in 1942 in his description of open reduction of LeFort fractures.[2] The information re ₊uired for full anatomical reconstructions can only be provided by detailed computed tomograms, which must be correlated with physical examination. It is now appropriate that the pattern of segmentation and displacement within each anatomical area of the face be defined and need to guide individualized exposure and fixation. Computed tomograms must be analyzed to determine the anatomical areas involved by the injury and the degree of injury as manifested by comminution and displacement.

Low, mid, and high "energy" injuries are identified for each anatomical location (Figure 2). The "energy" is the term used to describe the appearance of segmentation and displacement in the CT scan and does not relate to the actual multifactorial conditions which produce the appearance such as injury strength and bone strength. In the case of the low "energy" injury, there is little displacement and little need for sophisticated fixation. In many cases, either no or a simple treatment is required. Mid energy injuries in each anatomical region demonstrate mild to moderate displacement, and include a broad range of injury severity. Simpler treatments do not properly address these injuries. The approach to their reduction and fixation utilizes standard incisions on exposure, reduction and plate and screw fixation. The majority of the fractures analyzed are categorized and effectively treated as "mid energy" injuries. A small number of injuries observed in each anatomical region are classified as "high energy" injuries. In these patients, the degree of fragmentation, displacement and the instability following conventional approaches is sufficient to justify an extended open reduction proceeding well beyond local incisions. For these injuries, multiple surgical approaches to all the buttress articulations of a regional bone are utilized to confirm alignment and to provide fixation.

ANATOMICAL AREAS OF THE FACE

The Zygoma (Table I)

Low energy zygoma fracture (18%) demonstrate little or no displacement (Figure 3). They are incomplete through at least one articulation with the fracture relatively stable by virtue of the incomplete fracture. Frequently, the incomplete fracture is at the zygomaticofrontal suture. Minor degrees of displacement do not justify an open reduction.

Mid energy zygoma fractures (77%) demonstrate at least complete fractures at all buttresses (Figure 4a&b). With increasing injury severity, comminution at the zygomaticomaxillary buttress and inferior orbital rim are observed. Injuries in this group are managed by a standard open reduction of the zygoma, utilizing two anterior approaches: (1) a lower eyelid subciliary skin muscle flap and (2) intraoral exposure of the articulation of the zygoma with the maxillary alveolus utilizing a gingivobuccal sulcus incision. For selected injuries, a percutaneous approach to anterior zygomatic arch stabilization may be applied in this group.

The majority of zygomatic fractures in the mid energy designation are satisfactorily treated by these anterior approaches alone. The zygomaticofrontal suture, although providing the strongest bone for fixation, alone provides the poorest anatomical guide to the reduction. When increasing comminution is observed within the mid energy group, confirmation of anatomical reduction requires simultaneous visualization of the multiple anterior articulations of the zygoma, achieving a "best result" comparing malar projection and inferior orbital rim level with the contralateral side. A particularly helpful guide to reduction of the zygoma involves visualization of the alignment with the greater wing of the sphenoid in the lateral orbit.

High energy zygoma fractures are infrequently observed as an isolated injury. Only 5% of isolated zygomatic fractures correspond to this designation. They are characterized by comminution in the greater wing of the sphenoid in the lateral orbit (Figure 5), extensive zygomatic arch fractures which extend back to the glenoid fossa. Segmentation of the external angular process of the frontal bone may be observed. When the continuity of the zygomatic arch is destroyed, landmarks for anterior projection and stability for malar eminence support are withdrawn. The cheek is

depressed and midfacial width increases. Lateral and posterior displacement of the arch require, for confirmation of anatomical alignment and for reconstruction, a full exposure of the arch and of the multiple articulations of the zygoma with adjacent bones. A complete reconstruction of the zygomatic arch is required to align and stabilize the forward projection of the malar eminence; exposure of the lateral and deep orbit are required. Such exposures have been previously recommended by Manson[9] and Jackson.[13]

It should be noted that high energy "zygoma" fractures more frequently accompany LeFort or pan facial fractures as a segment of these injuries (see LeFort fractures). Less commonly, a high energy zygoma fracture exists as an isolated injury and requires a coronal incision for exposure and fixation.

Nasal Fractures (Table II)

Nasal fractures are identified as having both lateral and posterior displacement.[3,23] Injuries are best classified, however, by posterior displacement. The degree of posterior displacement corresponds to comminution in the nasal bones, septum and frontal process of the maxilla.

In low energy injuries (28%) (Table II), the fractures are predominantly unilateral with an absent or incomplete contralateral fracture (Figure 6). These injuries involve only the thin, broad segment of a unilateral distal nasal bone and the nasal septum. Septal fractures are predominantly in a reverse "C" shape.

Mid energy nasal injuries (66%) demonstrated bilateral injuries of the nasal bones and septum (Figure 7). The displacement usually consisted of both lateral and posterior dislocation. Simpler injuries were managed by closed reduction and nasal splint application. More extensive injuries demonstrated increasing saddling of the dorsum and telescoping of the septum. In more extensive injuries, fractures are not only bilateral, and extend into the proximal portion of the nasal bones but also involve the frontal processes of the maxilla). Usually, nasal bone fractures are more extensive on one side as compared to the other. The most common mistake in treatment was not thoroughly completing incomplete fractures of the usual bones and the septum by thorough closed manipulation. The nose should be able to be freely deviated and stand stable in any direction if incomplete fractures have been properly mobilized.

High energy nasal fractures (6%) are characterized by bilateral fractures extending to involve the pyriform margin of the maxilla (Figure 8). Fractures involving the pyriform rim and frontal process of the maxilla are the transition to more extensive injuries extending outside the nasal skeleton, such as the nasoethmoidal orbital fracture with involvement of the entire medial orbital rim. In more extensive but isolated nasal injuries, the use of closed reduction and an external nasal splint do not provide sufficient alignment or stabilization of fracture fragments. High energy nasal injuries are best managed either by the application of soft padded transnasal bolsters (to minimize nasal width and improve nasal height) by an open reduction of the pyriform rim and perhaps dorsal nasal bone grafting. Open fractures benefit from open reduction. In more extensive fractures, the septum may benefit from being "centralized" with wires at the anterior nasal spine and at the dorsal nasal bones. In cases with severe telescoping of the septum resulting in saddling of the nasal dorsum, immediate bone grafting may be indicated.

The Nasoethmoidal Orbital Region *(Table III)*

In its simplest form, a nasoethmoidal orbital fractures is defined as a fracture involving the lower 2/3's of the medial orbital rim with the potential for canthal ligament displacement. Unilateral or bilateral fractures are seen, and the fracture frequently extends to adjacent anatomical areas. Low energy injuries (18%) (Table III) demonstrate unilateral fractures isolating one medial orbital rim as a single segment which was minimally displaced inferiorly and undisplaced superiorly (Figure 9). Displacement of nasoethmoidal orbital fractures is first observed at the inferior orbital rim and pyriform aperture. In these injuries, the degree of periosteal continuity (as assessed on bimanual examination)[24] is often sufficient to provide enough stability to avoid a full open reduction. The "greensticked", minimally displaced fracture corresponds to the incomplete fracture of the zygoma previously described, where the fracture is incomplete at the zygomaticofrontal suture. In the low energy nasoethmoid orbital fractures, the fracture is incomplete at the junction of the frontal process of the maxilla with internal angular process of the frontal bone. Open reduction of these injuries is accomplished by subciliary and gingivobuccal sulcus incisions alone, reducing and stabilizing only the displaced regions. A unusual bilateral fracture may be present as a single segment ("monoblock") bilateral nasoethmoidal orbital injury.

Mid energy nasoethmoidal orbital fractures (72%) demonstrate displacement at the frontomaxillary suture, and may be unilateral or bilateral (Figure 10). They are characterized by a single, double or triple segment medial orbital rims where the segmentation does not divide the bone providing canthal ligament insertion. Exposure requires combined coronal, subciliary and gingivobuccal sulcus incisions to reduce and stabilize the articulations of the frontal process of the maxilla with adjacent bones. In selected cases local incisions or a laceration might be utilized for more limited fractures. The canthal ligament should not be detached in the reduction. Plate and screw fixation of the inferior ends of the frontal process of the maxilla is performed, but is not extended along the medial orbital rim. The medial orbital rim is stabilized to the frontal bone.

High energy nasoethmoidal orbital injuries (10%) demonstrate comminution of the medial orbital rim extending within the canthal ligament insertion (Figure 11a,b). In these cases, the segment of bone to which the canthus is attached is sufficiently comminuted that canthal stripping is required to stabilize the small pieces of the medial orbital rim. Plate and screw fixation along the medial rim is performed after the fragments are linked with wires to achieve a stable framework.

LeFort Maxillary Fractures (Table IV)

Low energy injuries (9%) (Table IV) are characterized by incomplete or minimally displaced fractures, similar to other areas (Figure 12). In this group, one observes simple fractures in the posterior and medial aspects of the maxillary sinuses unilaterally at the LeFort I level with "buckle and fissure" fractures (respectively). The anterior maxillary walls and pterygoid plates seem uninvolved on CT scans. These injuries are characterized by malocclusion without maxillary mobility. They are satisfactorily treated by intermaxillary fixation and elastic traction to restore normal occlusion.

Mid energy LeFort maxillary fractures (85%) have two common patterns presumably relating to the angle of impact. Direct frontal impacts (1) produce a basic LeFort II fracture pattern often extending through the nasoethmoidal area (Figure 13). More extensive injuries produce comminuted fractures separating a LeFort I segment within the LeFort II pattern, extending into the nasoethmoidal area and frontal sinus. Lateral impacts (2) demonstrate a unilateral zygomatic fracture accompanying a LeFort II or a LeFort I and II combination of fractures, depending on the severity of injury forces. The

confirmed zygomatic fracture, if of the high energy type, requires a coronal incision. Frequently, a hemi-nasoethmoidal orbital fracture is present of the mid or low energy type as described previously. 2% of LeFort fractures were unilateral (hemi LeFort) fractures. The alveolar segment of the maxilla is split in 15% of patients.

High energy LeFort fractures (6%) demonstrate excessive comminution in either the upper and/or lower midface (Figure 14). Extreme lateral and posterior displacement of the zygomatic segments may be observed with diffuse orbital segmentation. The LeFort I level and palate may be comminuted. In the most severe upper midface injuries, the bilateral nature of the extreme fractures of the frontal bone, nasoethmoidal orbital areas, midface and zygomas produce "traumatic hypertelorism" (Figure 15a,b,c). These rare fractures account for the most unstable and challenging fractures in the LeFort group.

Maxillary Alveolus

Simple alveolar fractures, low energy, were observed either as an isolated fracture or as a part of a LeFort midface fracture. Simple isolated fractures are stabilized by a dental arch bar and acrylic splint, with or without open reduction at the LeFort I level. Mid energy fractures usually split the maxillary alveolus sagitally and are usually seen as a portion of a LeFort midface injury.[26] In these patients, the palatal split parallels the midline suture of the palate. Open reduction of the fracture in the palatal vault stabilizes the width of the upper jaw, precisely establishing an index to the width of the entire lower face in more extensive fractures (Figure 16). Open reduction of the pyriform aperture, zygomaticomaxillary and nasomaxillary buttresses at the LeFort I level is also utilized. High energy fractures involving the maxillary alveolus demonstrate both sagittal and transverse palatal fractures isolating the maxillary tuberosity as a separate segment. These tuberosity fractures involve the posterior aspect of the lateral maxillary dentition. They are stabilized by buttress reduction at the LeFort I level, linking alveolar fragments with an extension of buttress plate and screw fixation (Figure 17), and or with an open palatal roof approach. An acrylic palatal splint may be used either temporarily as an index to proper occlusal alignment or for additional stabilization in highly segmented maxillary alveolar fractures.

Mandibular Fractures (Table V)

Mandibular fractures vary in anatomic location and are frequently multiple. Horizontal and vertical segments of the mandible are defined. Low energy impacts (43%) (Table V) produce minimally displaced, single fractures. Intermaxillary fixation alone or open reduction may be required depending on displacement, fracture location and muscular forces.

Mid energy impacts produce a multiply fractured mandible accounting for 47% of the mandibular fractures observed (Figure 18a,b). Double or triple fractures are seen with marked displacement confined to the horizontal mandible. Open reduction is required for fractures in the horizontal portion of the mandible. Ramus fractures are of a low energy type and may be treated with closed reduction and intermaxillary fixation. The low energy nature of the vertical mandibular fracture is the basis for this energy classification.

High energy mandibular fractures (10%) involve 3 - 6 segments (Figure 19a,b). Usually, bilateral subcondylar fractures or condylar fracture dislocations are observed in combination with comminuted fractures of he horizontal (anterior) portion of the mandible (symphysis or parasymphysis area). Complicated fractures require intraoral open reduction of fractures in the horizontal portion of the mandible and benefit from fracture reduction in the ramus condylar and subcon⟨   ⟩ regions, utilizing retromandibular and pre-auricular incisions. This complete 3-dimensional reconstruction is necessary for restoration of lower facial height, projection and for limiting lingual rotation of lateral mandibular segments. Displacement increases facial width at the mandibular angles and produces a lingual cant in the mandibular dentition.

Frontal Bone (Table VI)

Low energy injuries are simple linear fractures (60%) simultaneously involving the frontal bone and cranial base with little displacement (Figure 20). Frequently, observation alone is adequate. Frontal sinus fractures, even through simple require confirmation of nasofrontal duct patency and evaluation for posterior wall involvement (Figure 21).

Mid energy fractures (31%) demonstrated fractures dislocation involving one or two of the three areas of the frontal portion of the skull (Figure 22a,b). The frontal skull

is divided into central (frontal sinus) and lateral (frontal-temporal orbital) areas. Simple fractures extend within one of these areas, defined laterally and superiorly by cranial sutures and inferiorly by the orbital rim and frontal sinus margins. A common presentation involves dislocated fractures extending within a lateral or a central region. In more extensive fractures both a lateral and central region are simultaneously involved.

High energy frontal bone fractures (9%) involve both of the lateral and also the central regions of the frontal skull (Figure 23). They demonstrate extreme displacement. Rigid fixation of the supraorbital "bar" for anterior projection and the use of temporal fossa alignment to establish upper facial width are essential components of an anatomic reduction.

THE ORBIT

Low energy fractures demonstrate simple linear or circular blow out" fractures of the floor or medial orbital wall. Less commonly, the fracture may be a "blow in" fracture.[27] This fracture type is most frequently noted in the orbital roof (Figure 22), with inward displacement.

Mid energy orbital fractures may be isolated to the middle portion of the orbit but are usually accompanied by fractures of the orbital rim. Three types of rim fractures may be identified: zygomatic, supraorbital, or nasoethmoidal-orbital. The internal portion of the orbital fracture in these injuries commonly involves at least 2 orbital wall areas (such as floor and medial wall) (Figure 24) and is adequately stabilized by bone grafts supported by intact bone at the margins of the defect and at the rim. Anatomic alignment of internal orbital bone grafts is based on utilizing intact bone, at the margins of the defect. Intact posterior landmarks and restored rim position provide anatomical guides to the positioning and angulation of intraorbital bone grafts.

High energy orbital injuries are those in which there is extreme destruction of multiple segments of the rim and internal orbit. Frequently, these injuries are circumferential (3 or 4 wall). In these cases, it is difficult to adequately stabilize internal orbital bone grafts. The use of titanium mesh, extending as a structural support in the orbit, is indicated to convert the internal orbital fracture to a simpler injury. It is otherwise difficult to provide stability for small bone grafts where much of the internal

supporting bone has been destroyed. The most critical area for bone graft support in circumferential orbital injuries is the inferio-medial portion of the internal orbit.

Orbital fractures require true axial and coronal sections for the most precise evaluation. Reconstructed images do not consistently provide the best format for analysis of the displacement.

SUMMARY

Experience with facial injuries provides generalizations on common fracture patterns observed in computed tomograms. Fractures are first designated by their anatomical location and then classified by the pattern of comminution and displacement as low, mid or high "energy" injuries. In each case, physical exam correlates and the exposure and fixation required parallel the fracture pattern which is called the "energy" of the injury as revealed in the CT scan. Current application of exposure and techniques of craniofacial fixation are applied individually to each anatomic location. The use of standard incisions for facial fracture reduction (the coronal, the subciliary skin muscle flap, the gingivobuccal sulcus incision, the intraoral mandibular gloving incision, and the external retromandibular and preauricular incision) are related to use in fracture treatment according to the "energy" pattern observed in these fractures (Figure 25).

These data relate to blunt facial injuries observed in vehicular accidents. The pattern of fractures from other injuries may be different, and perhaps will be skewed toward less severe fractures. The emphasis of this discussion should be on the special treatment required for high energy injuries.

In high energy injuries, a dramatic potential for facial deformity exists. Facial proportions following high energy injuries demonstrate reduced anterior projection, reduced facial length and increased facial width. These deformities are only prevented by a complete anatomic exposure throughout each anatomical region extending fully to adjacent bones exposing all critical buttresses of a regional area both for confirmation of alignment and for fixation. Localized exposures, adequate for mid energy injuries, when utilized for these high energy injuries are inadequate for both alignment and fixation. Reconstruction begins with exposure of intact cranial or cranial base landmarks (posterior frame) and then assembles the anterior facial frame. Internal defects are bone grafted.

The use of computed tomograms allows the most complete identification of both subtle and extensive fractures. Our knowledge of the sequelae of facial injuries has been greatly increased by this information. Examples of "subtle" injuries "discovered" on CT scan include the incomplete LeFort maxillary fracture[25] and the fracture of the glenoid fossa which accompanies some zygomatic fractures. Such information supplements our knowledge of causes of impaired mandibular motion, pain or subtle malocclusion.

The identification of the degree of fragmentation in facial injuries has resulted in refined guidelines for exposure and fixation appropriate to the degree of injury. A nasoethmoidal fracture, incomplete at the fronto-maxillary suture is adequately reduced and properly stabilized by inferior approaches alone. For mandibular fractures, the multiply fractured mandible displays marked tendency to rotation of lateral mandibular segments and to open bite. These deformities are minimized by a full open reduction indexing the proper width and rotation of the mandibular dentition to the maxilla; anatomical continuity of ramus and condylar segments assists restoration of height and

projection, and acts to prevent open bite with release of intermaxillary fixation.

These guidelines provide a practical framework in which to plan facial injury treatment. The classification of any set of data requires somewhat arbitrary divisions which relate in part to personal preference and experience. They will, like any treatment protocol be continuously refined by personal experience as more precise imaging techniques are developed, and as increasing experience with rigid fixation of fractures is obtained.

TABLE I – ZYGOMATIC FRACTURES

LOW	18%
MID	77%
HIGH	5%

TABLE II – NASAL FRACTURES

LOW	28%
MID	66%
HIGH	6%

TABLE III – NASOETHMOID FRACTURES

LOW	18%
MID	72%
HIGH	10%

TABLE IV – LeFORT FRACTURES

LOW	9%
MID	85%
HIGH	6%

TABLE V – MANDIBULAR FRACTURES

LOW	43%
MID	47%
HIGH	10%

TABLE VI – FRONTAL BONE

LOW	60%
MID	31%
HIGH	9%

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FIGURE LEGENDS

Figure 1

Buttresses (vertical, right; horizontal, left) of the craniofacial skeleton.

Figure 2

Low (left), middle (center) and high (right) "energy" fractures of the zygoma.

Figure 3

CT of low energy zygoma fracture. A minimally displaced orbital floor fracture is also seen.

Figure 4 a and b

Two mid-energy zygoma fractures. In mid "energy" zygoma fractures, complete fractures and increasing comminution are observed at the anterior buttress articulations. The zygomaticomaxillary buttress and inferior orbital rim demonstrate the first comminution. The greater sphenoid wing, arch and frontal process are then involved. The zygomatic fracture shown has anterior arch displacement. Midfacial width is not increased; the medial displacement of the arch may be managed with anterior approaches.

Figure 5 a and b

In high "energy" zygoma fractures, comminution is observed at all buttress articulations including the posterior zygomatic arch. Lateral (increased midfacial width) and posterior displacement of the zygomatic arch (a,b) require a coronal incision for arch stabilization. Anterior approaches are still required for the other buttresses.

Figure 6

A unilateral distal nasal fracture, low "energy" type.

Figure 7

A mid energy, bilateral nasal fracture.

Figure 8

High "energy" nasal fractures demonstrate bilateral involvement, comminution and displacement extending throughout both nasal bones and the nasal septum extending into the pyriform margin of the maxilla. Open reduction or external soft bolsters yield the best results.

Figure 9 a, b, c

A low "energy" nasoethmoidal orbital fracture is incomplete at the junction of the frontal process of the maxilla with the internal angular process of the frontal bone (a). Reduction and fixation may be accomplished with inferior approaches at the lower orbital rim and gingivobuccal sulcus.

Figure 10

A mid "energy" nasoethmoidal orbital fracture with complete and bilateral dislocation of the medial orbital rim at all buttress articulations. Moderate segmentation is observed except within the area of the canthal insertion. Canthal detachment from bone is not required. A coronal incision is required when displacement involves the articulation of the internal angular process of the frontal bone with the frontal process of the maxilla.

Figure 11

(a) A high "energy" nasoethmoidal orbital fracture with extreme comminution and dislocation. The bone providing canthal ligament attachment is shattered and is the basis for this energy designation. Complete reconstruction (b) requires canthal ligament disinsertion, bone reduction and a transnasal canthopexy.

Figure 12

Incomplete LeFort fractures are best characterized by "buckle" and "fissure" fractures observed at the LeFort I level in the medial and posterior aspects of the maxillary sinuses. The pterygoid plates seem uninvolved. These fractures are managed by intermaxillary fixation alone.

Figure 13

(a,b,c) A mid "energy" LeFort fracture demonstrates comminution through the LeFort I level (c) involving the anterior, medial and posterior maxillary walls (b) and pterygoid plates (b). Open reduction and rigid fixation are required.

Figure 14

(a,b,c) In high "energy" LeFort fractures, the upper and lower midface demonstrates high energy injuries. The remainder of the midface fractures may show less involvement. In this patient, the maxillary alveolus and LeFort I level are severely comminuted. (High energy lower midface injury). The zygomas, orbits and nasoethmoidal-orbital areas demonstrate mid and high energy fractures.

Figure 15

(a,b,c) Lateral displacement of both globes secondary to dislocation of the frontal bone, zygomas and nasoethmoid regions produce traumatic hypertelorism. This is a high energy upper midface injury.

Figure 16

Open reduction of a sagittal fracture of the palate (Figure 14c) in the roof of the mouth.

Figure 17

Reduction of a posterior alveolar ("tuberosity fracture") with a plate posterior to the zygomaticomaxillary buttress.

Figure 18

(a,b) Two mid "energy" mandible fractures. (a) demonstrates mild and (b) demonstrates severe displacement confined to the horizontal portion of the mandible, each with two fractures. The horizontal mandible requires an open reduction with plate and screw fixation at each fracture.

Figure 19

(a,b) High "energy" mandible fractures demonstrate three or more segments with involvement of the horizontal and vertical mandible. Fracture dislocations require an open reduction in both horizontal and vertical mandibular areas. In the vertical segment, fracture displacement and instability benefit from an open reduction including condylar/subcondylar areas.

Figure 20

Undisplaced frontal skull fracture just to left of midline.

Figure 21

Linear fracture of right frontal skull and posterior wall of frontal sinus.

Figure 22

(a,b) Mid "energy" injuries of the frontal bone frequently involve the central and lateral areas of the frontal bone simultaneously and demonstrate moderate segmentation and displacement. Two examples are seen.

Figure 23

High "energy" injuries of the frontal bone demonstrate involvement of both lateral and the central areas of the frontal skull. Comminution is extreme. Alignment and projection are improved by assessing bilateral temporal bone and anterior cranial fossa alignment.

Figure 24

A "three wall" (lateral, floor and medial) orbital fracture is seen on the left and a two wall (medial and floor) is seen on the right.

Figure 25

Five incisions which allow exposure of all critical areas of the facial skeleton.

Figure 1

Figure 2

Figure 3

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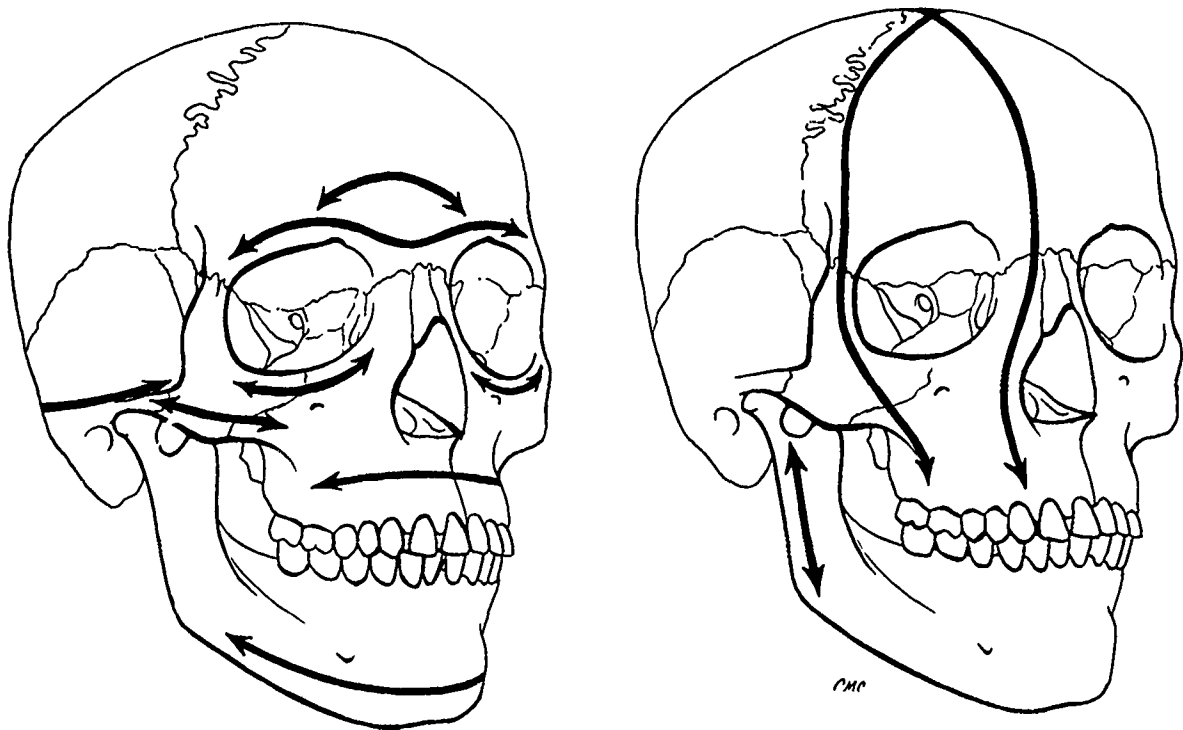
Figure 4a

Figure 4b

Figure 5a

Figure 5b

Figure 6

Figure 7

Figure 8

Figure 9a

Figure 9b

Figure 9c

Figure 10

Figure 11A

Figure 11B

Figure 12

Figure 13

Figure 14A

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Figure 14B

Figure 14C

Figure 15A
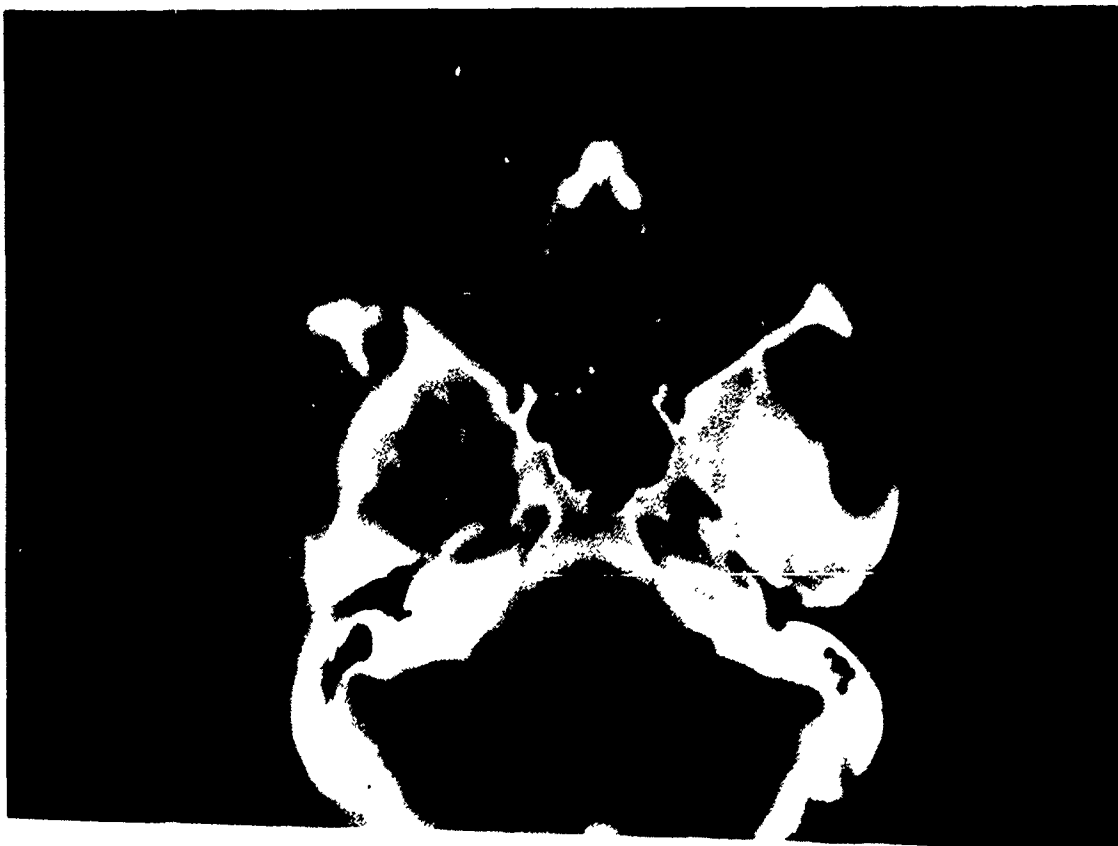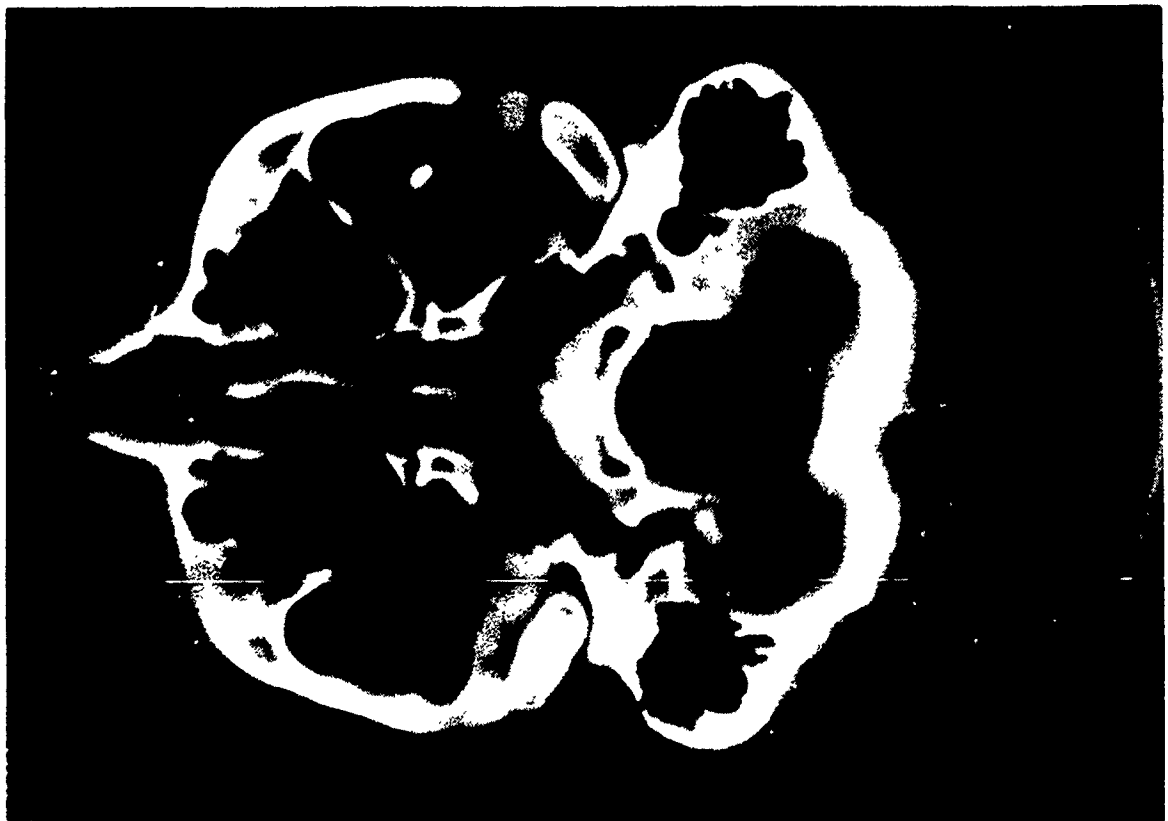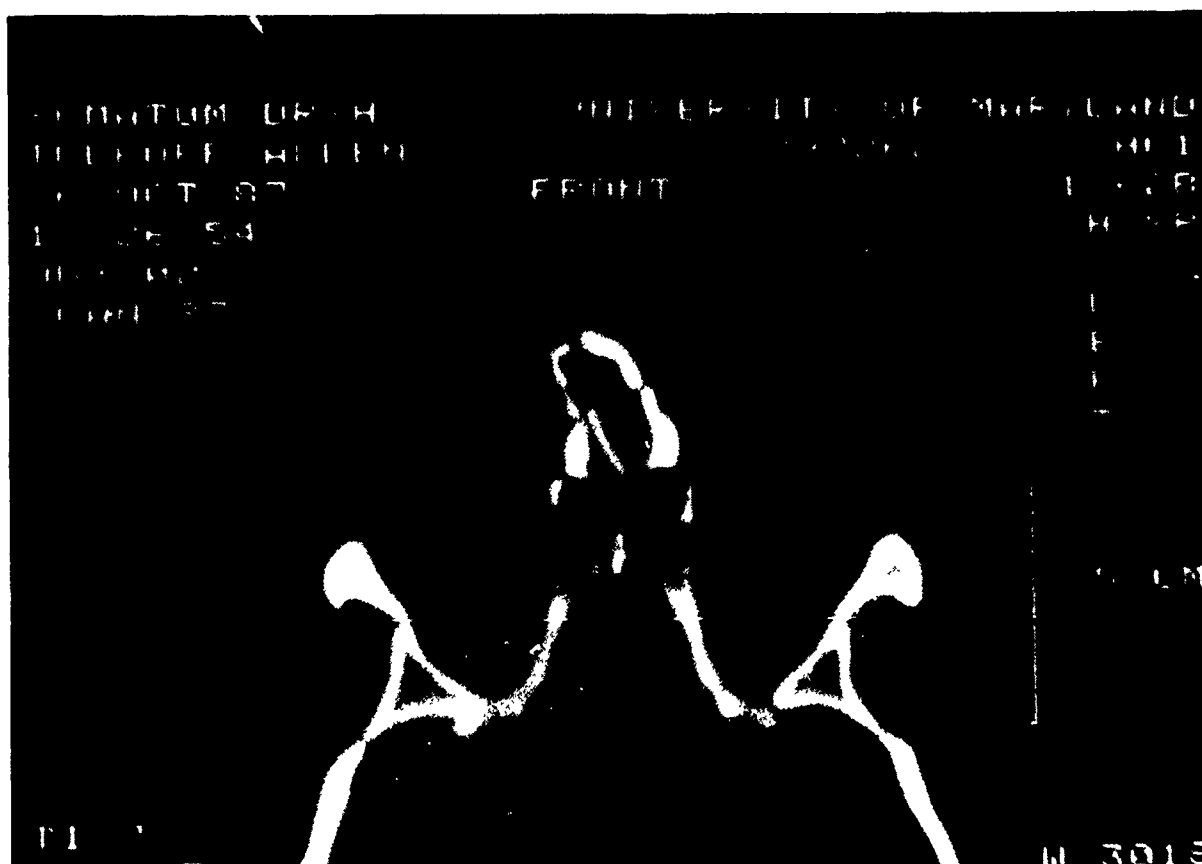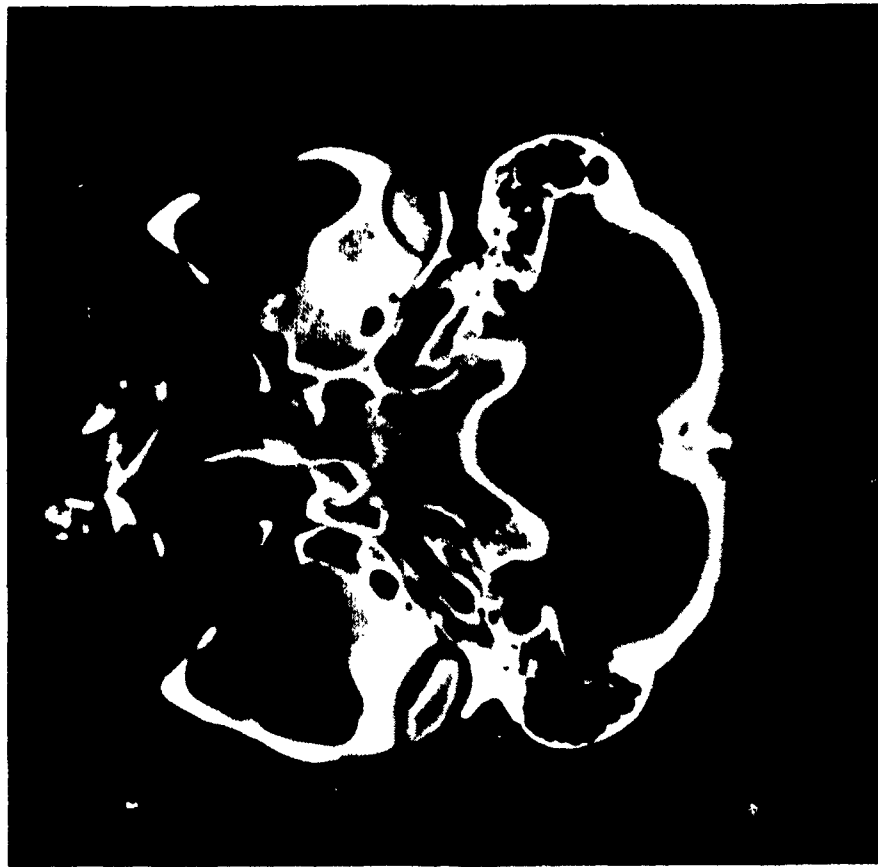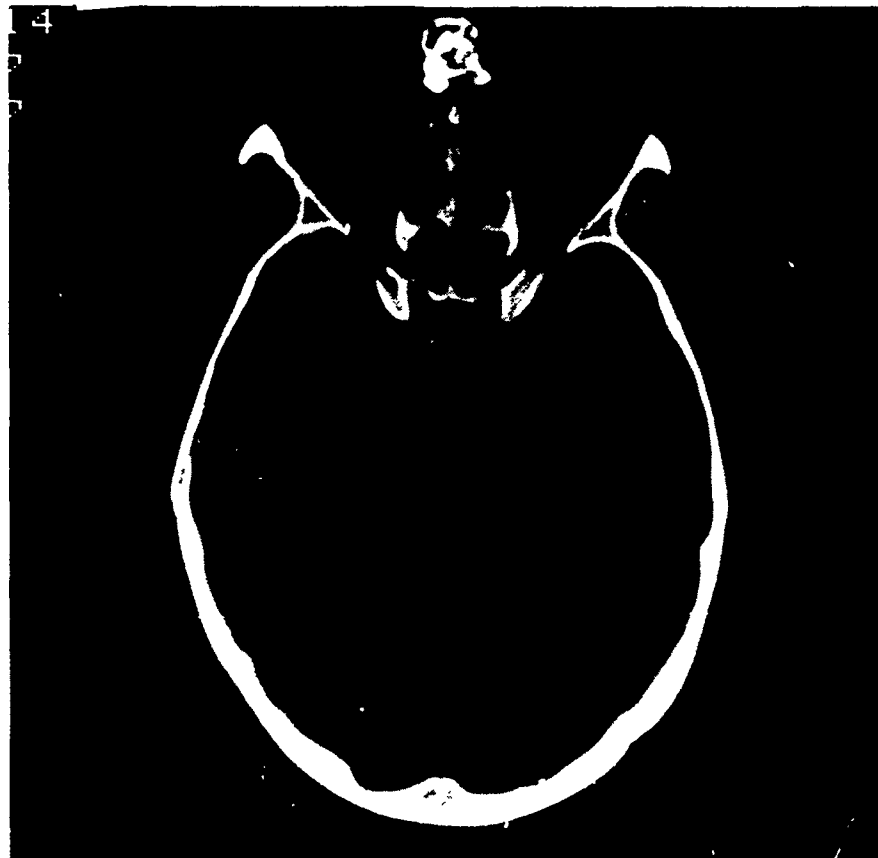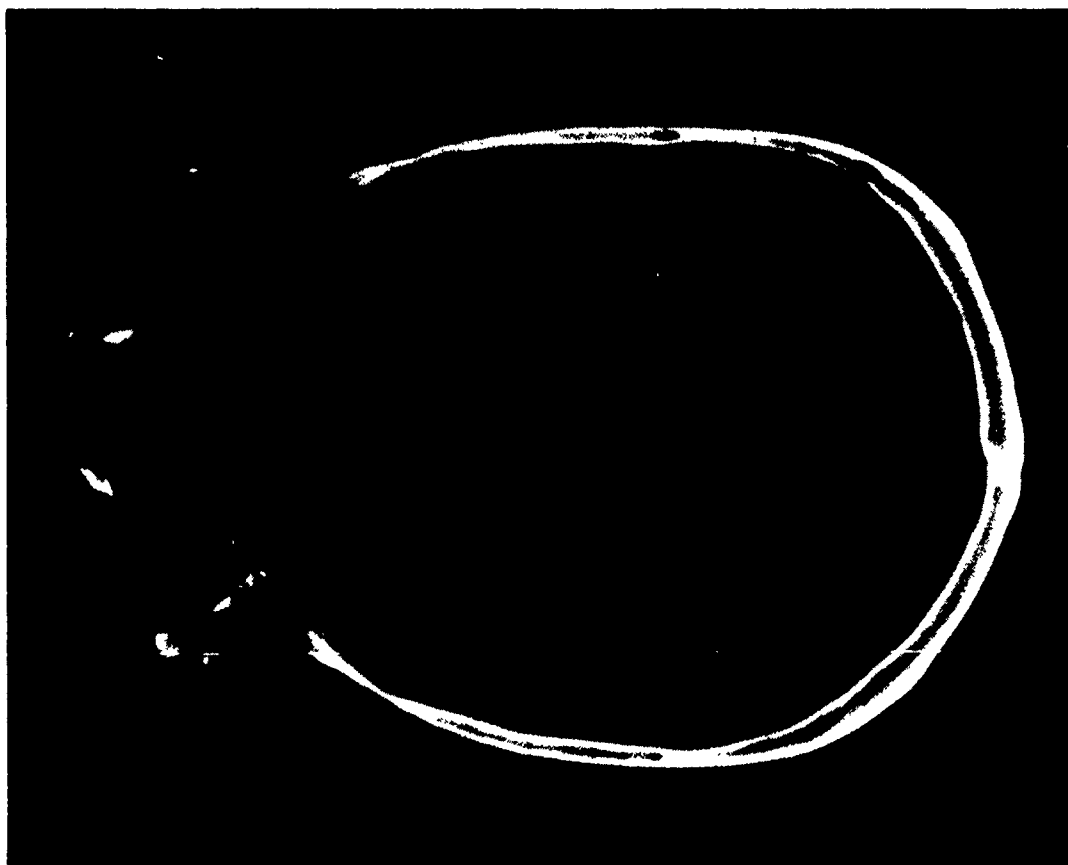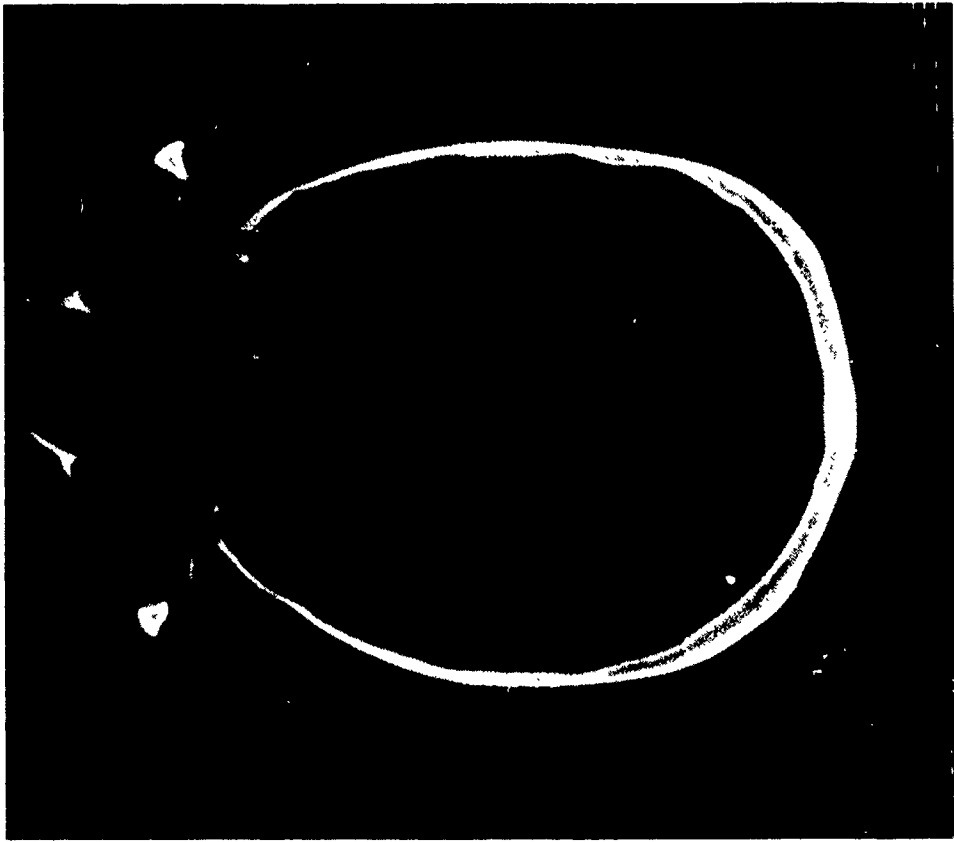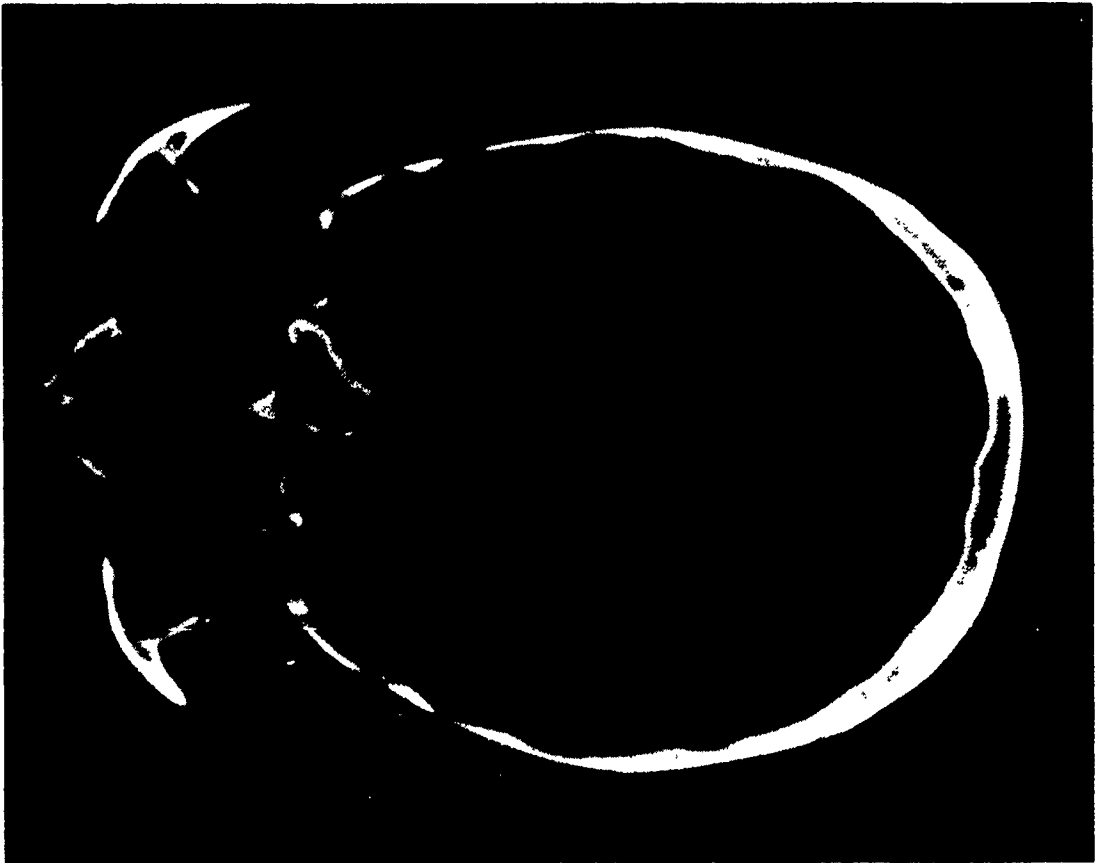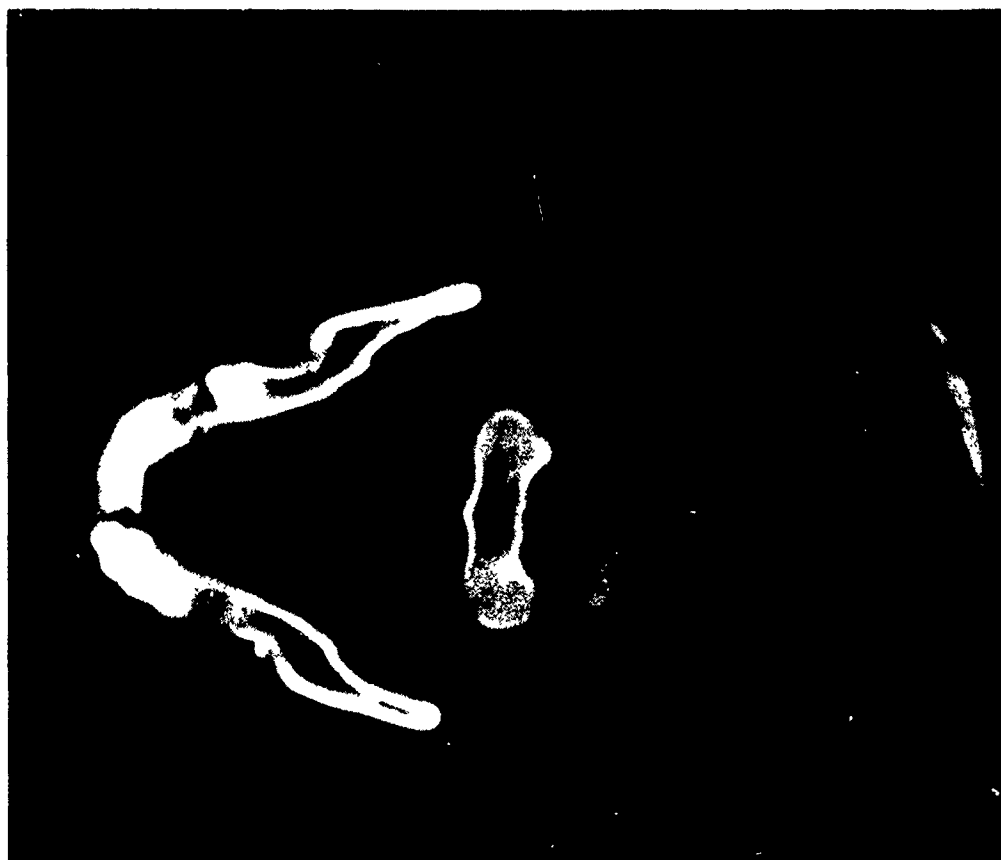
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Figure 15C

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Figure 16

Figure 17

Figure 18A

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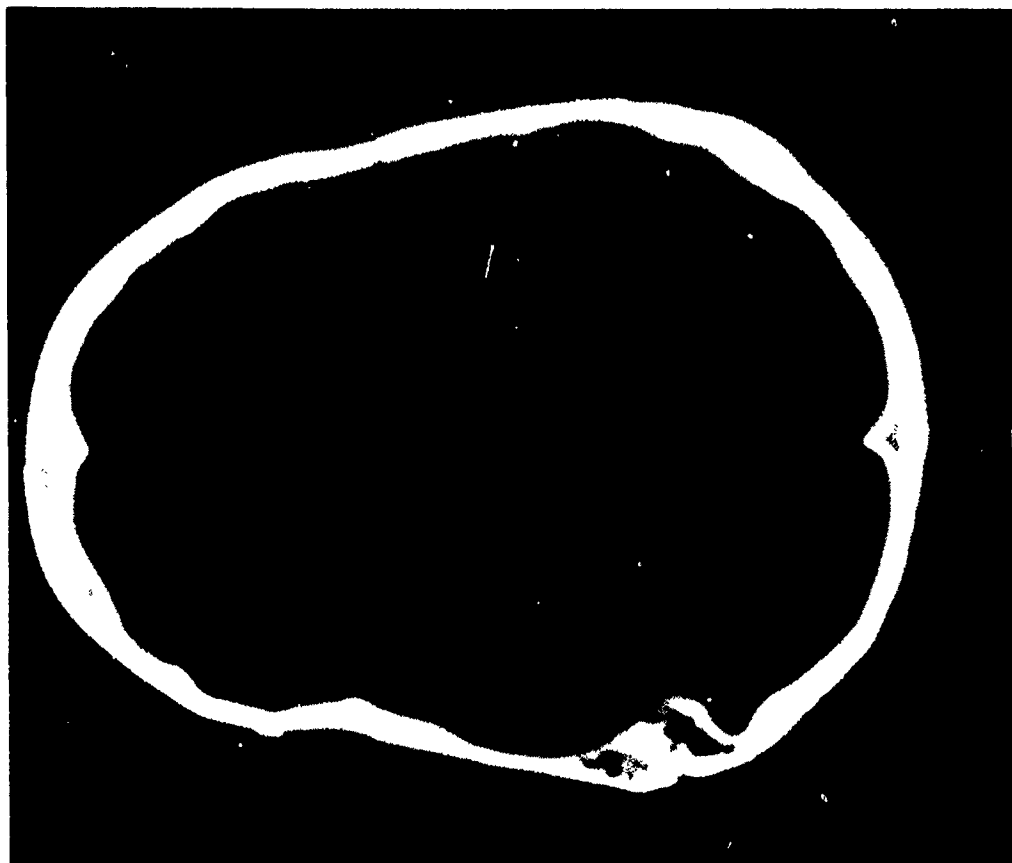
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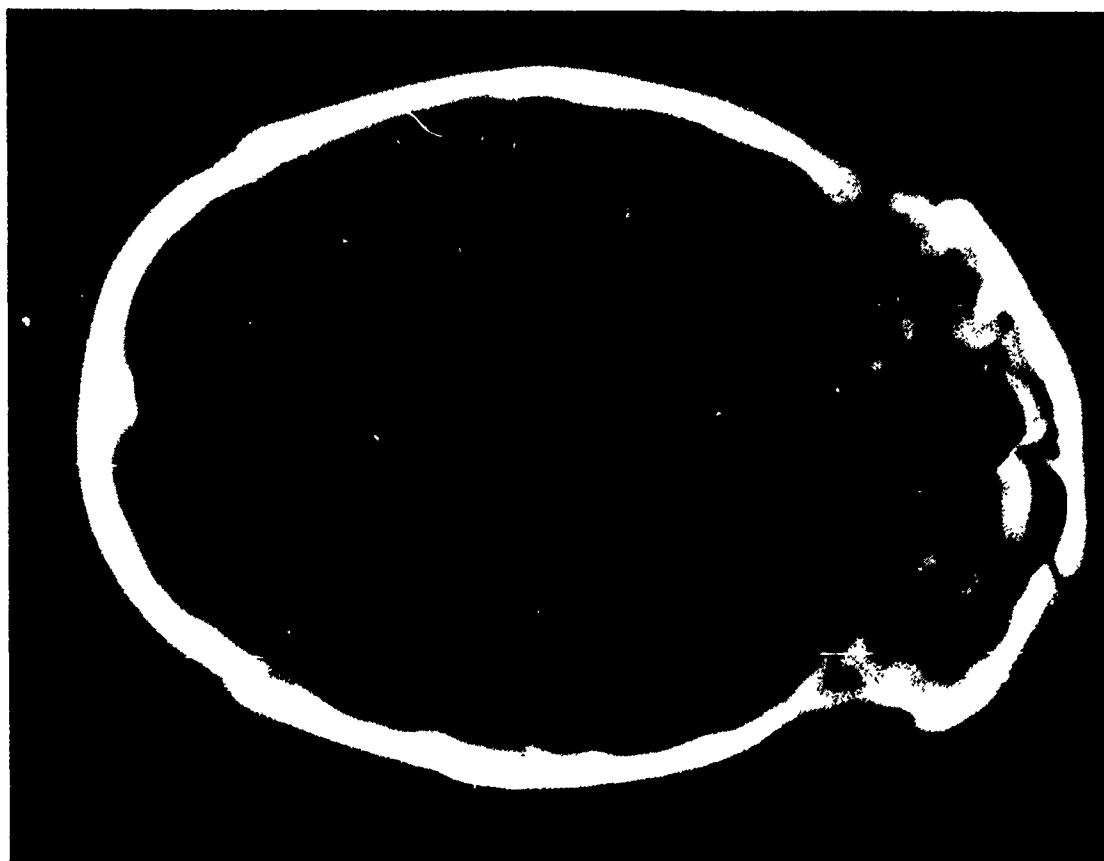
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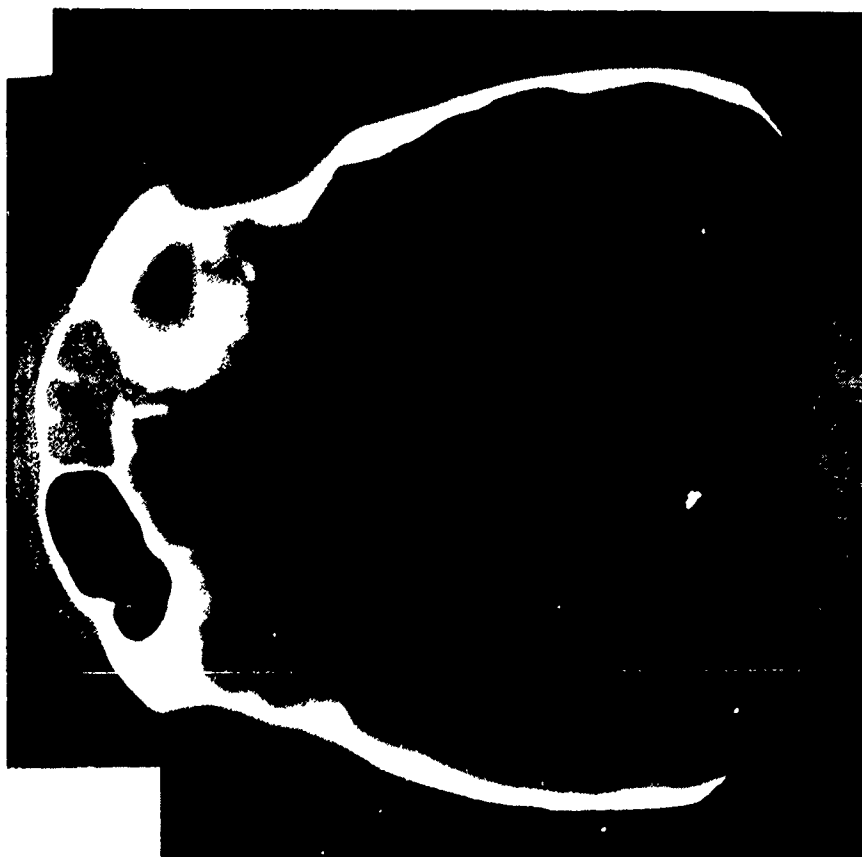
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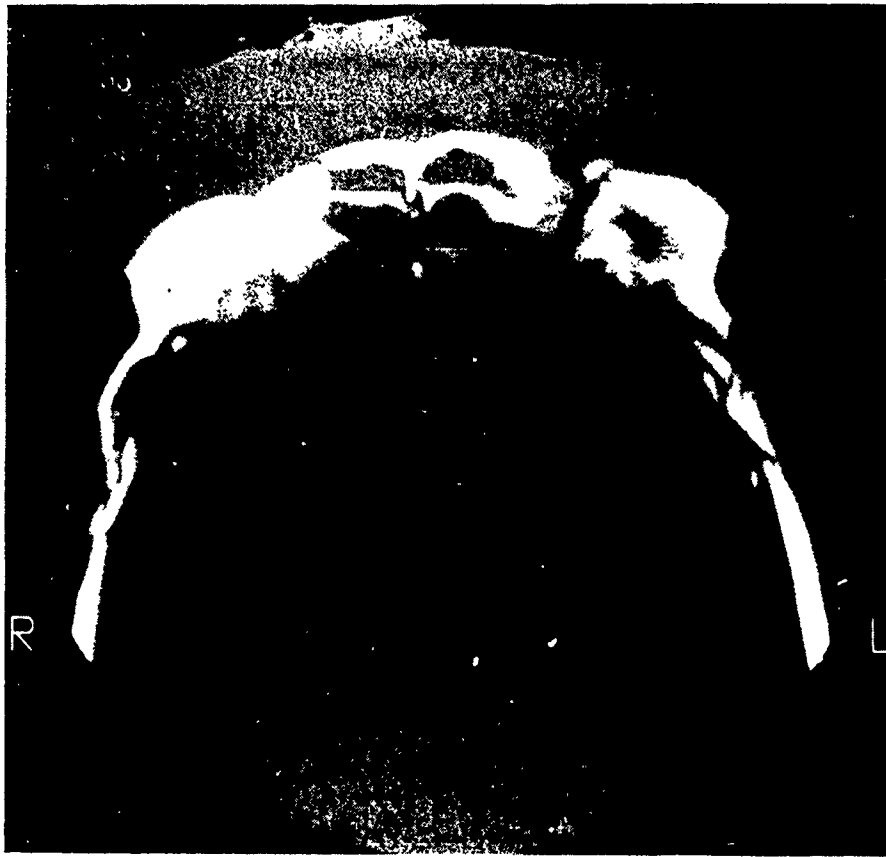
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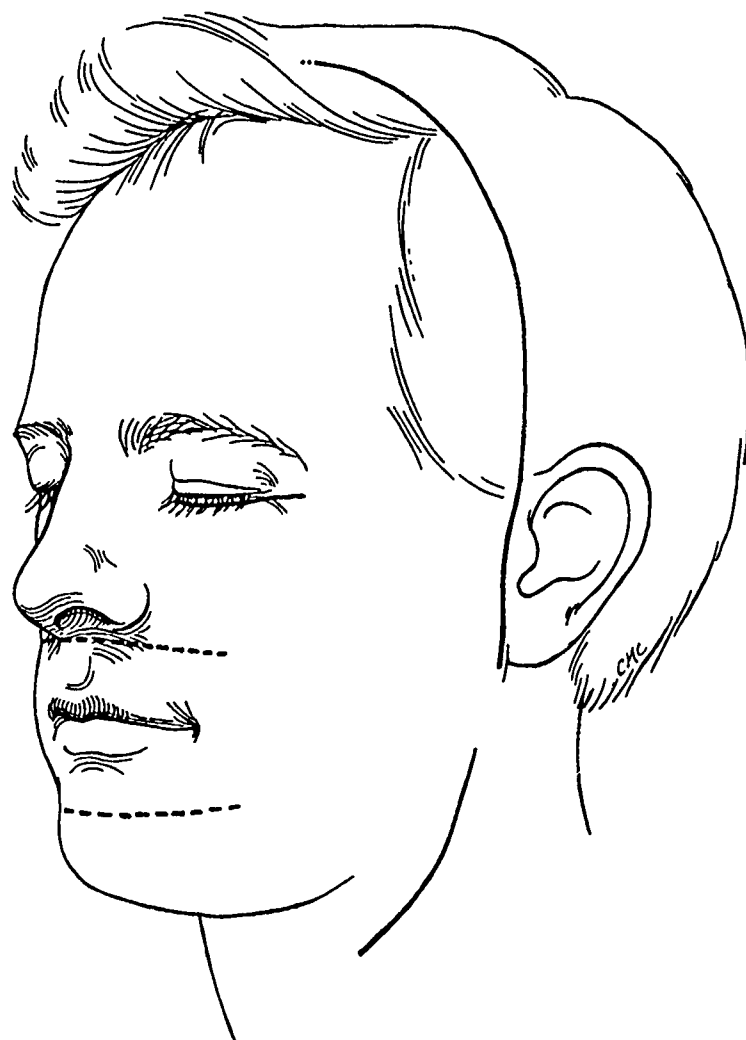
Figure 24

Figure 25

"HISTOMORPHOMETRY OF SURGICAL, BIOMECHANICAL AND GRAVITATIONAL EFFECTS AT THE MACROSCOPIC AND MICROSCOPIC LEVELS"

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ABSTRACT

Histomorphometry is stereological quantification of histological sections. Fully mineralized histological sections may be evaluated with stains, polarized light and/or microradiography. Use of intravital mineral and cellular labels provides a unique physiological perspective of osseous response to environmental challenges such as surgery, mechanical loading and weightlessness. Demineralized 3 µm bone sections are preferable for autoradiography, immunocytochemistry and nuclear volume morphometry. Histomorphometric data can be collected via a broad array of image analysis devices. Relatively simple, manual techniques with routine ocular grids and specific bone preparations are often preferable because the data is easier to interpret. Percent volume, specific surface (bone surface perimeter or area), linear intercept and nuclear volume morphometry are particularly valuable histomorphometric methods.

INTRODUCTION

Histomorphometry often reveals important physiological parameters that are not obvious with routine microscopy. Static parameters of bone architecture, like volume of mineralized tissue and fraction of cortical vs. trabecular bone, are measured directly from sections or block faces. Microradiographic images provide an index of relative mineralization. Physiological inference of dynamic parameters is best achieved in intravitally labeled preparations. The anabolic chronology of bone is determined by administering a series of bone labels that mark the bone appositional or mineralization front at known time intervals. Cell proliferation indices are assessed by injecting DNA labels such as 'H-thymidine or bromodeoxyuridine. Anabolic bone markers, microra-

diography and DNA labeling are important adjuncts for the modern histomorphometric analysis of bone.

Macroscopic assessment of internal architecture, such as relative distribution of cortical and trabecular bone, is performed on tissue sections or block faces. The most common volumetric approach is the point-hit method (Cavalieri Principle). A grid or similar geometric reticule is randomly oriented over a representative section of a bone. Within the periosteal domain of the bone, the percentage of intersections ("hits") over cortical bone, trabecular bone and nonosseous tissue are determined. Volumetrically, the relative prevalence of each tissue type is directly proportional to the number of hits divided by the total number of grid interceptions sampled (Fig. 1). Bone surface area is determined by randomly orienting a series of parallel lines of known interval over the section of interest and counting the number of intersections with the bone surface (Fig. 2).

Microscopic structure of bone is assessed similarly. If the specimens are labeled with an intravital label like tetracycline, a wide range of physiological inference is possible. A typical double label protocol for clinical use involves administration of tetracycline (250 mg/kg x 2 d), an interlabel period of 7-10 d, a second dose of tetracycline for 2 d and a label burial period of 2-5 d. Double labels allow for accurate measurement of the advancing mineralization front. This parameter is often referred to as the "bone apposition rate." However, the initial event in bone formation is deposition of unmineralized matrix or osteoid. Only matrix markers such as ^{3}H-proline labeling of matrix collagen actually measure bone apposition. Calcium seeking molecules like tetracycline are fluorescent markers of the mineralization front. Under steady state conditions bone "apposition" and "mineralization" rates are directly related, but they are not same thing because lamellar bone matrix must mature for about 7-10 days prior to accepting mineral.

PHYSIOLOGICAL METHODOLOGY

Morphology of bone is well described, but its physiology is elusive because of the technical limitations inherent in the study of mineralized tissues. Bone function is often inferred from structure. However, morphology is only a snapshot in time of the physiological events operative at that instant Time markers (bone labels) and physio-

logical indices (S-phase cells) are essential for defining bone dynamics *in situ*. Systematic investigation with advanced methods have defined new concepts of clinically-relevant bone physiology.[1-7]

Physiological interpretation of bone morphology at the microscopic level requires specially adapted methods: 1) <u>mineralized sections</u> are an effective means of accurately preserving structure and function relationships, 2) <u>microradiography</u> assesses mineral density patterns in the same sections, 3) <u>fluorescent labels</u> such as tetracycline permanently mark all sites of bone mineralization at a specific point in time (anabolic markers), 4) <u>polarized light birefringence</u> detects the preferential orientation of collagen fibers within bone matrix, 5) <u>autoradiography</u> detects radioactively tagged precursors (nucleotides, amino acids, etc.) used to mark physiological activity, and 6) <u>nuclear volume morphometry</u> differentially assesses osteoblast precursors in a variety of osteogenic tissues.

Mineralized Sections

Fully mineralized specimens are superior to routine demineralized histology for most critical analyses of teeth, periodontium and supporting bone. There is less processing distortion and both the inorganic mineral and organic matrix can be studied simultaneously. For tissue-level studies, 100 μm thick sections are appropriate because they can be studied with multiple analytical methods. Cellular detail and resolution of bone labels are considerably enhanced by reducing the thickness of the section to <25 μm. Specific stains are useful for enhancing contrast of both cellular and extracellular structures. The disadvantages of thin mineralized sections include more rapid quenching of bone labels and inadequate tissue density for microradiographic analysis.

Microradiography

High resolution images require polished sections about 100 μm in thickness. Differential x-ray attenuation reveals that new bone is less mineralized than mature bone. Newly formed bone matrix requires about a week of maturation to become mineralizable osteoid. Osteoblasts then deposit up to 85% of the eventual mineral complement by a process referred to as primary mineralization.[6] Secondary mineralization (mineral maturation) completes the maturation process in about 8 months by a crystal growth process (Fig. 3). Since strength of bone tissue is directly related to mineral content, the

stiffness and strength of an <u>entire</u> bone depends on the distribution and relative degree of mineralization of its osseous tissue.[8] The initial strength of new bone is due to the cell-mediated process of primary mineralization, but its ultimate strength is dictated by secondary mineralization, the physicochemical process of crystal growth. This concept has important clinical value in dentistry. Fully mineralized lamellar bone, in steady state with respect to modeling and remodeling, is stronger and more resistant to loads than its woven and composite bone predecessors.[9,10] Postoperative loading of a dental implants and retention following active orthodontic therapy are clinical applications of this principle.

Fluorescent Labels

Administered *in vivo* calcium binding labels are anabolic time markers of bone formation. Histomorphometric analysis of label incidence and interlabel distance is an effective method for determining the mechanisms of bone growth and functional adaptation. Because they fluoresce at different wavelengths, six different bone labels can be used in the same tissue: 1) tetracycline (10 mg/kg, bright yellow), 2) calcein (5 mg/kg, bright green), 3) xylenol (60 mg/kg, orange), 4) alizarin complexone (20 mg/kg, red), 5) demeclocycline (10 mg/kg, gold), and 6) oxytetracycline (10 mg/kg, dull or greenish yellow). The labels are administered intravenously to dogs and intramuscularly to rabbits and rats. Tetracycline and oxytetracycline are available in commercial preparations for injection. Demeclocycline, calcein, xylenol and alizarin complexone are dissolved in a 2% sodium bicarbonate solution. The pH is adjusted to 7.4 and the preparation is filtered prior to injection. The multiple fluorochrome method, sequential use of a variety of different color labels, is a powerful method for assessing bone growth, healing, turnover, response to applied loads and functional adaptation to weightlessness (Fig. 4).

Polarized Light

Birefringence of polarized light and mineral density have particular biomechanical significance. The lamellar fringe patterns revealed with polarized light indicate the preferential collagen orientation within the matrix.[11] Most lamellar bone features alternating layers of collagen fibers at right angles resulting in intricate layers of

lamellae (Fig. 5). However, two specialized collagen configurations are noted in the same or adjacent osteons: 1) longitudinally aligned collagen - efficiently resists tension, 2) alternating or transverse lamellae - are particularly strong in compression, and 3) circumferential collagen fibers - are preferential support for torsion. It appears loading conditions at the time of bone formation dictate the orientation of the collagen fibers to best resist the loads to which the bone is exposed. The important point is bone formation can adapt to different loading conditions by changing the internal lamellar organization of mineralized tissue.

Autoradiography

Radioactive precursors for structural and metabolic materials are detected within tissue by coating histological sections with a nuclear track emulsion. Localization of radioactive disintegrations reveals the location of the radioactive precursors. Specific radioactive labels for proteins, carbohydrates or nucleic acids are injected at a known interval prior to tissue sampling. Qualitative and quantitative assessment of label uptake is a physiologic index of cell activity. The most common autoradiographic labels used in bone research are ^{3}H-thymidine labeling (Fig. 6) of cells synthesizing DNA (S-phase cells) and ^{3}H-proline labeling of newly formed bone matrix. 5–bromo–2–deoxyuridine (BDU) immunocytochemistry is a more recent nonradioactive method for labeling S-phase *in vivo*. It promises to be an important bone cell kinetic method of the future.

Nuclear Volume Morphometry

Nuclear size measurement is a cytomorphometric procedure for assessing the stage of differentiation of osteoblast precursor cells. Preosteoblasts have significantly larger nuclei than their committed osteoprogenitor precursors or their osteoblast progeny. Careful cytomorphometric assessment of nuclear size has proven to be an effective means for determining the relative differentiation of PDL and other bone lining cells. The increase in nuclear size (A'—>C) that occurs as committed osteoprogenitor (A') cells differentiate to preosteoblasts (C cells) is the key, rate limiting step in osteoblast histogenesis (Fig. 7).[12,13] Cell distribution studies in rat molar PDL have demonstrated that less differentiated osteogenic cells (A+A') are paravascular, i. e.,

within 20 μm of a blood vessel. Preosteoblasts are concentrated >30 μm away from blood vessels.(Fig. 8).[14] Nuclear volume morphometry of bone lining cells labeled with ^{3}H-thymidine has proven to be a powerful histomorphometric method for assessing the cell kinetics and cell population dynamics of bone.

TYPES OF BONE

Particularly when exposed to surgical and mechanical challenge, osseous tissue is often a mixture of different types of bone (Fig. 9). Each type has physiological advantages and disadvantages. Thorough histomorphometric analysis requires a careful distinction between the different types of bone tissue.

WOVEN BONE is highly variable in structure, relatively weak, disorganized and poorly mineralized. It serves a critical wound healing role by: 1) rapidly filling osseous defects, 2) providing initial continuity for fractures and osteotomy segments, and 3) strengthening a bone weakened by surgery or trauma. Woven bone is not found in the adult skeleton under normal, steady state conditions. It is either compacted to form composite bone or is remodeled to lamellar bone.[5] These maturation processes are important aspects of orthodontic retention and the postoperative healing period following orthognathic surgery.

LAMELLAR BONE is a strong, highly organized and well mineralized tissue that composes >99% of the adult human skeleton. When new lamellar bone is formed, only a fraction of the total mineral component is deposited by osteoblasts during primary mineralization. Secondary mineralization is a physiochemical (crystal growth) process requiring many months.[6] Within physiological limits, strength of bone is directly related to mineral content.[8] In ascending order, the relative strengths for different histological types of osseous tissue are: woven bone < new lamellar bone < mature lamellar bone.[15] The bone of adult humans is almost entirely of the remodeled variety, i. e., secondary osteons and secondary spongiosa.[5] Clinically, it is important to remember that full strength of the lamellar bone supporting a dental implant is not achieved for up to a year after the device is placed.

COMPOSITE BONE is an osseous tissue formed by depositing lamellar bone within a woven bone lattice, i. e., cancellous compaction.[9] This is the most rapid means for producing relatively strong bone. Composite bone can sustain normal function for

152

a substantial period of time, but in man is ultimately remodeled to lamellar bone, i. e., secondary osteons or secondary spongiosa. Cancellous compaction, either to form primary osteons (fine compaction) or whorling bone (coarse compaction), is the usual mechanism of cortical bone growth. Composite bone is an important intermediary type of bone in the physiological response to orthodontic loading. This is usually the predominant osseous tissue for stabilization during the early process of retention or postoperative healing. When formed in the fine compaction configuration, the resulting composite of woven and lamellar bone is also known as a primary osteon. Although composite bone is high quality, load bearing osseous tissue, in long-lived creatures such as humans, it is eventually remodeled to secondary osteons.[6,15]

BUNDLE BONE is a functional adaptation of lamellar structure for attachment of tendons and ligaments. The major distinguishing characteristic is perpendicular striations of bundle bone called Sharpey's fibers. Distinct layers of bundle bone are usually seen adjacent to PDL along physiological bone forming surfaces.[6]

FORM AND FUNCTION

Bone adaptation to the mechanical environment occurs by altering: 1) mass, 2) geometric distribution, 3) matrix organization, and 4) lamellar collagen orientation. In addition to these adaptive mechanisms influencing bone formation, the mechanical properties of osseous structures are also influenced by maturation, function, aging and pathologic processes. Important histological indications of bone mechanical properties are: 1) degree of secondary mineralization, 2) mean bone age, 3) fatigue damage, and 4) loss of vitality (hypermineralization).[3]

Modeling and Remodeling

Both trabecular and cortical bone grow, adapt and turn over by two fundamentally distinct mechanisms: *modeling* and *remodeling*. Bone modeling involves independent sites of resorption and formation that change the form (shape and/or size) of a bone. However, bone remodeling is a specific coupled sequence of resorption and formation events to replace previously existing bone. Bone modeling is the dominant process in growth as well as in adaptation to applied loads (Fig. 4 and 5). We see *modeling* changes on cephalometric radiographs, but *remodeling* events which are usually present at the

same time are only apparent at the microscopic level. True remodeling is not usually imaged on clinical radiographs.[2] The mechanism for internal remodeling of dense compact bone is via axially oriented cutting/filling cones.[9]

Traumatic or surgical wounding usually results in intense but localized modeling and remodeling responses. Following a fracture or osteotomy, callus formation and resorption of necrotic osseous margins are modeling processes. However, internal replacement of necrotic cortical bone, such as a rigidly fixed osteotomy of the mandible or the interface of an endosseous implant, is a remodeling activity associated with the postoperative RAP (regional acceleratory phenomenon).[5,16] Following orthognathic surgery, orthodontists can take advantage of the intense postoperative modeling and remodeling activity to: 1) orthopedically position a maxilla with headgear or cervical support within a few weeks following a Le Forte I osteotomy, and 2) rapidly finish orthodontic alignment of the dentition. On the other hand, restorative dentists must avoid overloading dental implants until the RAP is complete and the secondary bone is mature.

Both modeling and remodeling are controlled by an interaction of metabolic and mechanical signals. Bone modeling is largely under the local control of functional and applied loads. Although remodeling responds to mechanical loads at the tissue level, it is largely under the systemic influence of metabolic mediators, like parathyroid hormone and 1, 25 vitamin D. Continual bone turnover provides a metabolically controllable flow of calcium out of and into the skeleton.

Structural and Metabolic Fractions

The metabolic calcium reserves of the body are primarily in trabecular bone and the endosteal half of the cortices. The structural fraction of cortical bone is the relatively stable outer portion of the cortex. The metabolic fraction is the highly reactive inner aspect (Fig. 10). Analogous to stainless steel wires, stiffness and strength of a bone are directly related to its diameter. Diaphyseal rigidity is rapidly enhanced by adding circumferential lamella at the periosteal surface. Even thin layers of new osseous tissue at the periosteal surface greatly enhance bone stiffness because it increases the shaft diameter. In engineering terms, rigidity of tubular bones is related to the second moment of the area.[17]

New bone added at the endosteal surface has little effect on strength. Structurally, long bones and the mandible are modified tubes, an optimal design for achieving maximum strength with minimum mass.[8] Within limits, loss of bone at the endosteal surface or within the inner third of the compacta has little effect on bone strength. The inner cortex can be mobilized to meet metabolic needs without severely compromising bone rigidity. This is the reason osteoporotic patients have bones with normal diameter but thin cortices. Even under severe metabolic stress, the body follows a cardinal principle: maintenance of maximal strength with minimal mass.[1] In histomorphometric analysis of bone, it is important to distinguish the metabolic from the structural fraction.

BIOMECHANICS

Histomorphometric methods have helped define mechanical loading that is consistent with skeletal health. Regular exercise helps build and maintain skeletal mass. Suboptimally loaded bones undergo disuse atrophy by increasing the remodeling frequency while inhibiting osteoblast formation. Under these conditions, trabecular connections are lost and cortices are thinned from the endosteal surface. Eventually the skeleton is weakened until it cannot sustain normal function. Atraumatic fractures of the hip, wrist or spine are classic signs of osteoporosis. Multiple crush fractures of vertebrae contribute to loss of height and a stooped posture commonly referred to as "dowager's hump".[1]

Regular hypertrophic exercise helps build and maintain skeletal mass by muscular and gravitational loading. Peak loads exceeding the minimal effective strain, about 0.25% deformation or 2500 microstrain (μE), induce anabolic bone modeling, usually bone formation at the periosteal surface. Streaming potentials are generated within loaded bone and displaced periodontium.[18] Loaded bones and teeth generate a unique separation of charge which is associated with a specific osteogenic response.[19]

Mechanical control of bone modeling and remodeling is related to strain history. Frost[20] has proposed the *mechanostat theory* in an attempt to unify the often conflicting effects of loads on bone modeling and remodeling. Subthreshold loading of <200 μE results in disuse atrophy, manifest as decreased modeling and increased remodeling. Physiological loading of about 200-2500 μE is associated with normal, steady state activity. Loads exceeding the minimal effective strain (about 2500-4000 μE) result in a

hypertrophic increase in modeling and concomitant decrease in remodeling. However, once peak strains exceed about 4000 µE, structural integrity of bone is threatened, i. e., pathological overload. Refer to Martin and Burr[11] for a recent review.

It is important for bones to maintain functional strains and flexure within the range of normal skeletal balance. When flexure exceeds the normal physiological range, bones compensate by adding new mineralized tissue at the periosteal surface. This is an essential compensating mechanism because fatigue resistance of bone is inversely related to the load (strain magnitude).[21] When loads less than 2000 µE, lamellar bone can withstand millions of loading cycles, i.e., more than a lifetime of normal function. However, increasing the cyclic load to 5000 µE (about 20% of the ultimate strength of cortical bone) produces failure in a thousand cycles. The latter is easily achieved in only a few weeks of normal activity. It is clear that repetitive overload at less than a fifth of the ultimate strength of lamellar bone (25,000 µE) can lead to skeletal failure, stress fractures, shin splints, etc.

Clinical examples of pathological overload are well known. Shin splints and stress fractures are common for inappropriately trained athletes and military recruits. From a dental perspective, fatigue failure can result in periodontal clefting, alveolar recession, loss of implant integration, dental oblation (cervical ditching) and/or temporomandibular arthrosis. Therefore, optimal distribution of occlusal loads is an important objective of dental treatment because the high loads of oral function may exceed the body's ability to compensate. Much of the data for the biomechanical response of bone is derived form carefully designed histomorphometric studies.

GRAVITATIONAL EFFECTS

Gravitational loads have a substantial influence on normal skeletal physiology. Cell kinetic studies with DNA labels have been particularly effective in helping define the profound effects of simulated and actual weightlessness. Osteoblast differentiation leading to new bone formation is stimulated by mechanical loading[12] but is inhibited in microgravity.[13,22] The block in osteoblast histogenesis is the mechanical stress and/or strain mediated step to form a preosteoblast from a committed osteoprogenitor cell.

Space flight studies have established that gravity helps maintain skeletal mass.[23,24] A substantial portion of the physiological loading of the mandible is associated with

antigravity posturing. Because of the differential head posture of both rats and man, gravity tends to open the mouth. Muscle force is used to keep the mouth closed. Apparently, due to a lack of functional loading, growth of the rat mandibular condyle is inhibited in the microgravitational environment of space flight.[25] Nuclear volume morphometry is a well established method in gravitational biology.

ENDOSSEOUS IMPLANTS

A multiple fluorochrome histomorphometric study in dogs tested the anchorage potential of two prosthetic-type titanium implants: 1) a prototype endosseous device with a cervical post, asymmetric threads and an acid-etched surface, and 2) a commercially available implant with symmetrical threads (Brånemark, Nobelpharma, Sweden). Based on label incidence and relative number of new osteons in microradiographs, there was a higher rate of remodeling in the alveolar bone supporting the implant than in the basilar mandible.[26] Compared to titanium implants with a smooth surface, the degree of remodeling at the interface is greater for threaded implants placed in a tapped bone preparation.[15] This may be related to the increased resistance of threaded implants to torsional loads over time.[27]

The velocity of evolving second osteons (rate of movement through bone), determined by measuring the distance between initiation of labeling sites in longitudinal sections, was 27.7 ± 1.9 µm/day, mean $\pm$ SEM, n=4 (10 cutting/filling cones averaged for each dog). At this velocity, evolving secondary osteons travel about 1 mm in 36 days. Newly remodeled secondary osteons, formed within the experimental period of the dog study, contained an average of 4.5 labels (administered 2 weeks apart); the incidence of resorption cavities was about one-third the incidence of labeled osteons.[4] These data are consistent with a remodeling cycle of about 12 weeks in dogs compared to 17 weeks in man.[5,6] This relationship is useful for extrapolating dog data to human applications.

Eight Brånemark implants were placed bilaterally in the mandibles of four adult dogs. After an 8 week unloaded healing phase, transmucosal abutments were placed. After 2 week healing, a 3 Newton (306 g) load was applied in the sagittal plane for 12 weeks to half of the implants. The other half received no supplemental load. Osseous proximity to the endosseous interface of the Brånemark implants was evaluated

histomorphometrically in microradiographs of midfrontal sections. The interface was analyzed histomorphometrically at x100 using the linear intercept method as previously described.[4] Briefly, bone directly opposing the interface was classified as a "hit". Bone within 50μm of the implant surface was "near". If the nearest bone was >50μm from the surface of the implant, the sampled site was a "miss". The implants were divided into cervical (C), middle (M) and apical (A) regions for analysis. In general, the unloaded implants showed no regional differences, but the *loaded implants had more direct bone contact ("osseointegration") in the middle and apical regions* (p<0.05) (Figures 12 and 13). This application of the linear intercept method to microradiographs demonstrates an important histological relationship that has considerable clinical significance.

CONCLUSIONS

Modern physiological assessment of bone histology requires careful selection of intravital labels, microscopic techniques and histomorphometric methods. A well designed experiment utilizing simple, well focused methodology is often superior to an elaborate automated approach.

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FIGURE LEGENDS

Figure 1

The percentage of bone in the field of view is determined by counting the number of grid intersections lying over bone ("hits") in two usually perpendicular orientations. The two measures are averaged and divided by 100 to reveal that 20% of the field of view is bone.

Figure 2

Specific surface (Sv) is determined by counting the number of equally spaced line intercepts ("hits") on an ocular reticule that cross the bone surface. The grid or stage is rotated 90° between measures. The number of hits is averaged, multiplied by D (distance between the test lines) and $\pi/2$. Since D in this example is 100 μm, the bone perimeter is about 1.88 mm.

Figure 3

A microradiograph of rapidly remodeling cortical bone shows numerous new secondary osteons that are relatively radiolucent (primary mineralization) compared to the surrounding bone. Secondary mineralization is seen as increasing radiopacity radiating form the Haversian canal.

Figure 4

Ultraviolet illumination reveals the bone growth mechanism via a series of intravital bone labels administered at one week intervals. Active bone deposition (modeling) at the periosteal surface (p) produces numerous new primary osteons (*). New secondary osteons (s) are forming deeper in the compacta at the same time (remodeling). From Roberts *et al.* Calfornia Dental Association Journal 15(10):56, 1987.

Figure 5

Polarized microscopy of the same section viewed in figure 4 shows primary (*) and secondary (s) osteons forming simultaneously as growth occurs at the periosteal surface (p). Secondary osteons are distinguished by a scalloped border (arrow) which is the

resorption arrest line. From Roberts *et al.* Calfornia Dental Association Journal 15(10):56, 1987.

Figure 6

An autoradiograph of rat molar periodontium reveals a layer of ^{3}H–thymidine labeled osteoblasts adjacent to the periodontal ligament (P). Orthodontic loading induced a layer of new bone (N) on the surface of the original bone (O). A few labeled progenitor cells remain in the periodontal ligament.

Figure 7

The osteoblast histogenesis pathway is series of five kinetically distinct cell types (A, A', C, D and OB). There are two proliferation events shown as S–phase (S) and mitosis (M). The rate limiting step in osteoblast production is the mechanically mediated (stress and/or strain) increase in nuclear size to form a G_1 stage preosteoblast (C cells). Adapted from Roberts and Morey, American Journal of Anatomy. 174:112, 1985.

Figure 8

Less differentiated precursor (A) cells are a perivascular population. Committed osteoprogenitor (A') cells migrate away from blood vessels, differentiating to G_1 stage preosteoblast s (C cells). Following DNA synthesis, C cells become D cells, divide and migrate to the bone surface. Adapted from Roberts *et al.* Journal of Periodontal Research 22:456, 1987.

Figure 9

A demineralized section of human periodontium shows cementum (c), dentin (d) and periodontal ligament (P) of a tooth being moved to the right. Woven (*), lamellar (L) and bundle (B) bone are noted. A nearby marrow cavity (M) is evident ... From Roberts *et al.* California Dental Association Journal 15(10):54, 1987.

Figure 10

Ultraviolet microscopy of multiple fluorochrome labeled rabbit bone reveals a high rate of remodeling in the metabolic (M) fraction near the endosteal surface (left). The

structural (S) fraction near the periosteal surface is strengthened by new layers of lamellar bone.

Figure 11

Schematic diagram of osteoblast histogenesis showing that the stess and/or strain mediated step in preosteoblast fromation is inhibited by the microgravity of space flight. From Garetto *et al.* FASEB. Journal 4(1):25, 1990.

Figure 12

Bone intercept counts are shown for "unloaded"(no supplemental load superimposed on function) Brånemark implants place in the mandibles of dogs. Frontal sections reveal little difference in the amount of direct bone contact (Hits) in the cervical, middle and apical regions.

Figure 13

Bone intercept counts for "loaded" (3 N continous load in sagittal plane) Bråne- mark implants demonstrate more bone contact ("osseointegration") in the middle (strong trend) and apical regions (p<05) compared to similar "unloaded" implants (Figure 12).

VOLUME PERCENTAGE (V)

Roberts, Simmons, Brezniak
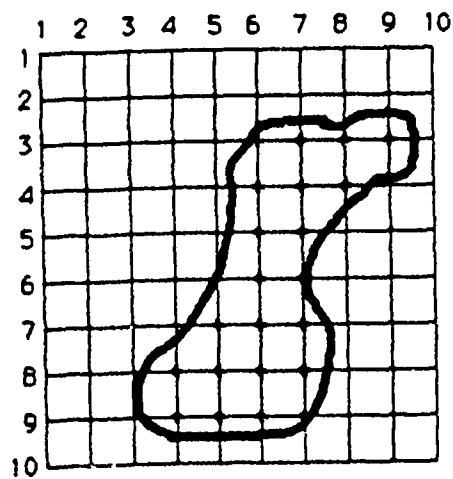
22 hits
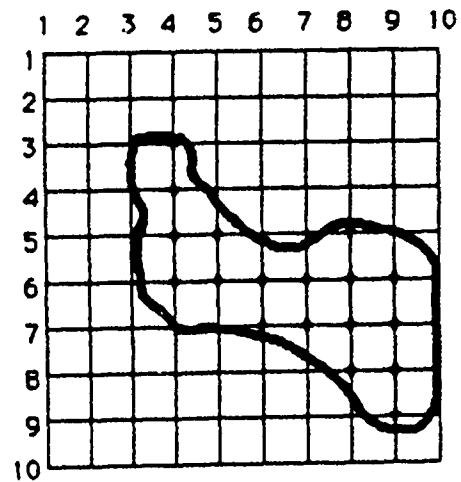
18 hits

$$V = \frac{22+18}{2} / 100 = 20\%$$

Figure 1

SPECIFIC SURFACE (Sv)
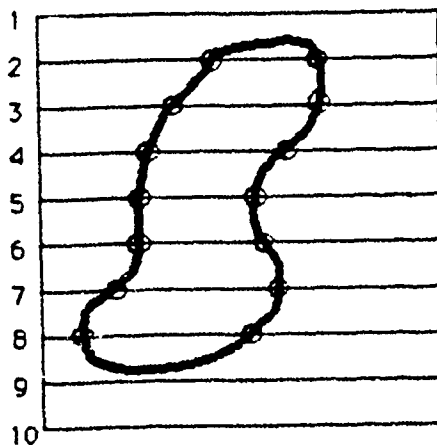
14 hits

10 hits

$$Sv = \frac{14+10}{2}(D)(\pi/2)$$

165

Figure 2

Figure 3

Figure 4

166
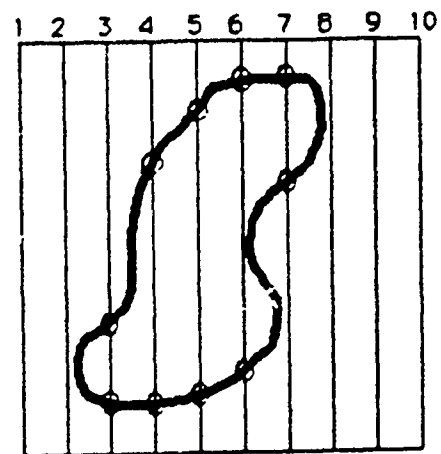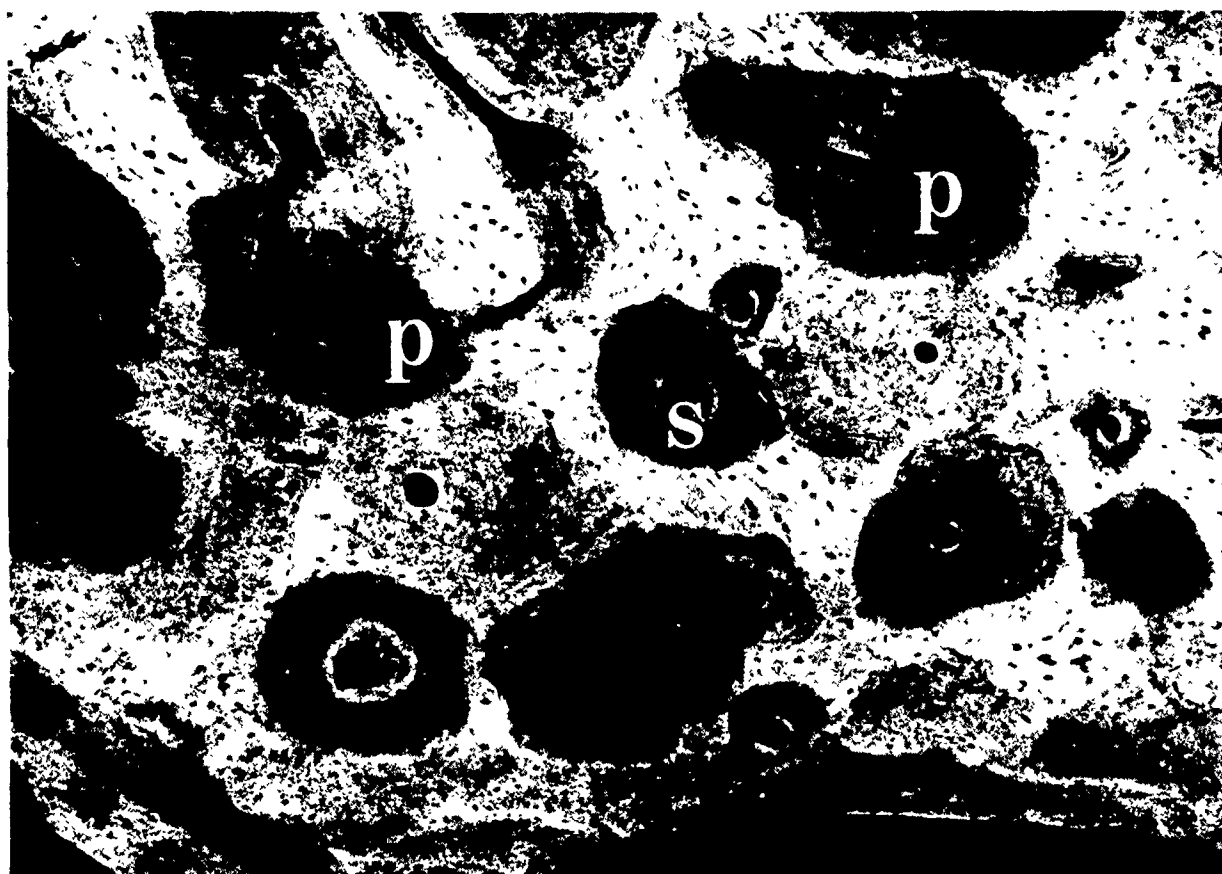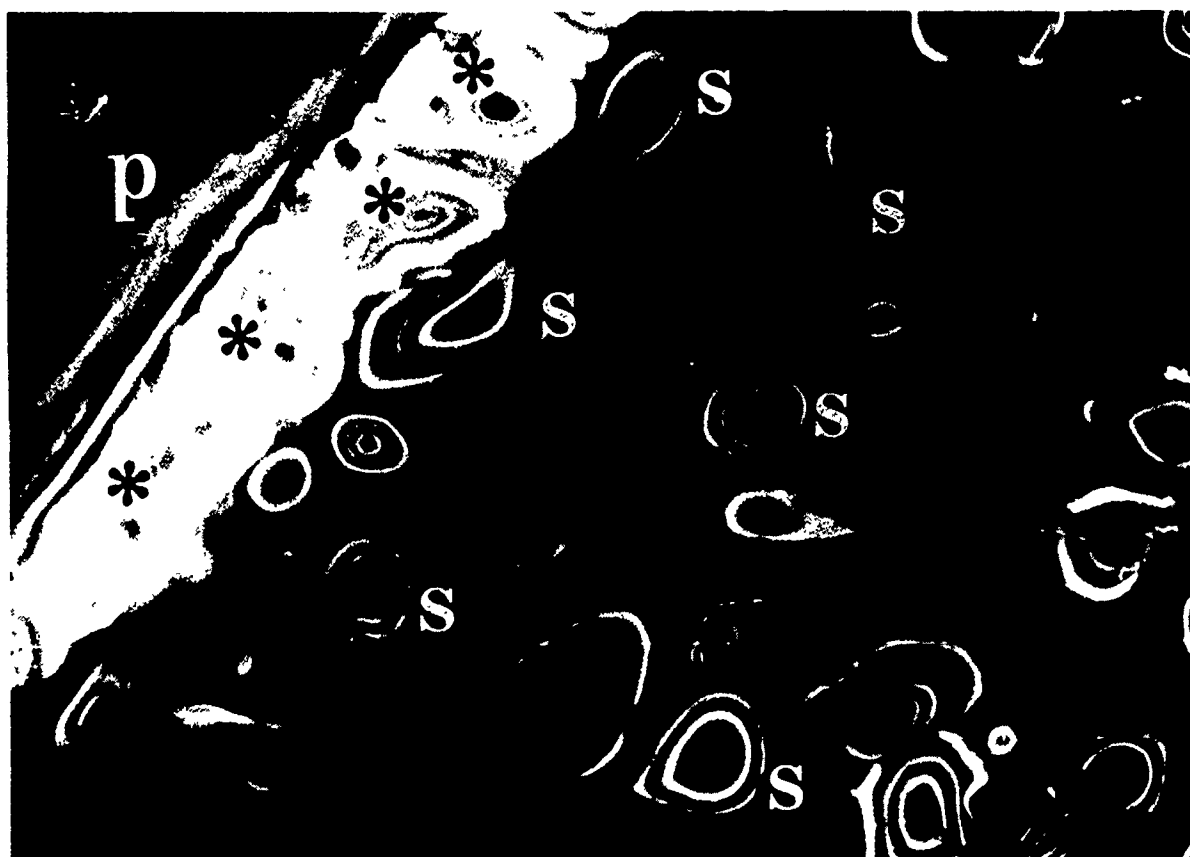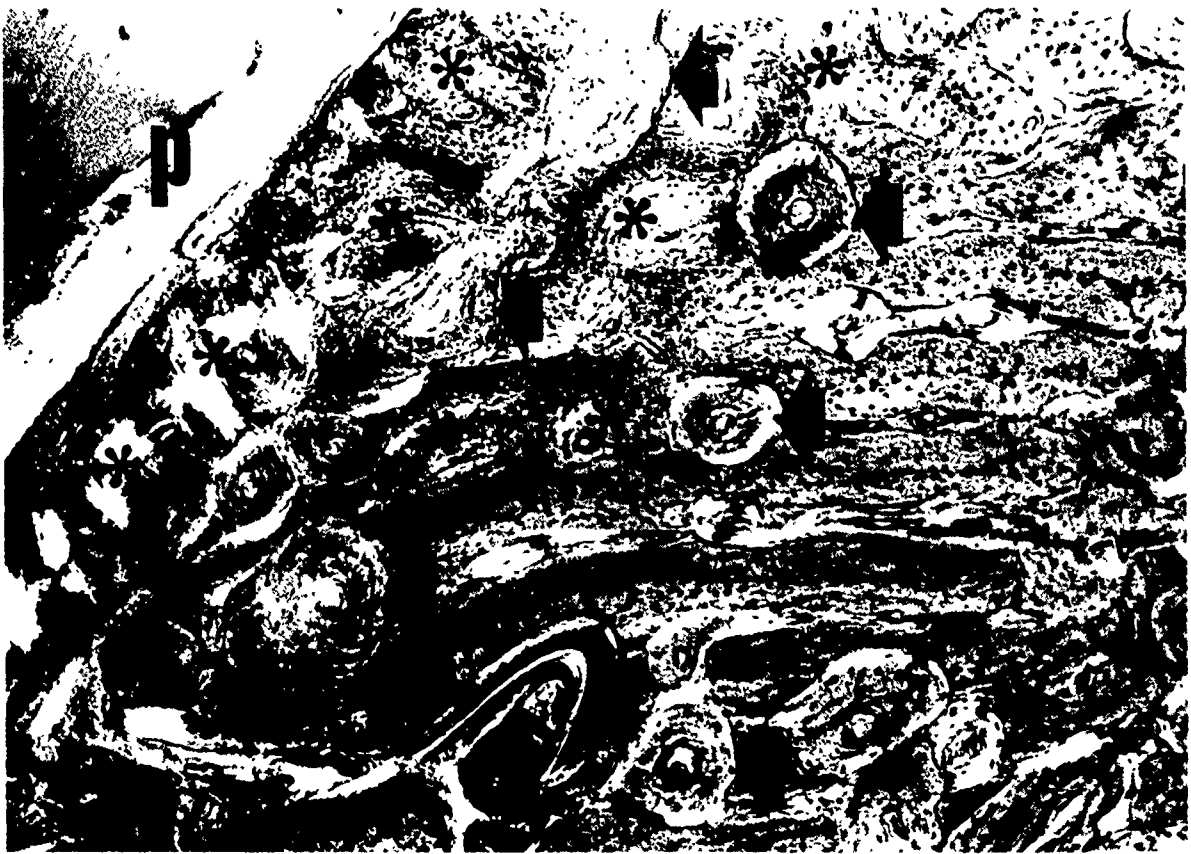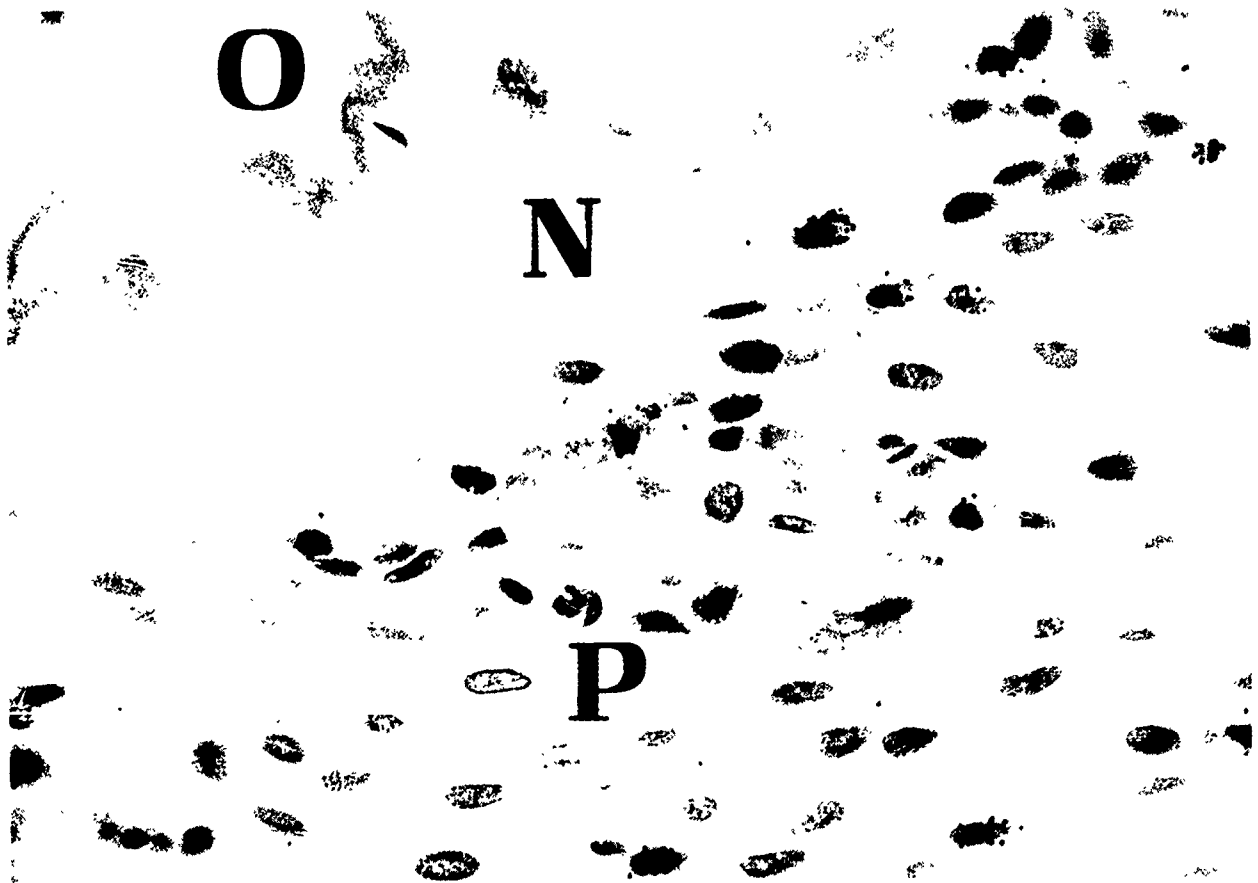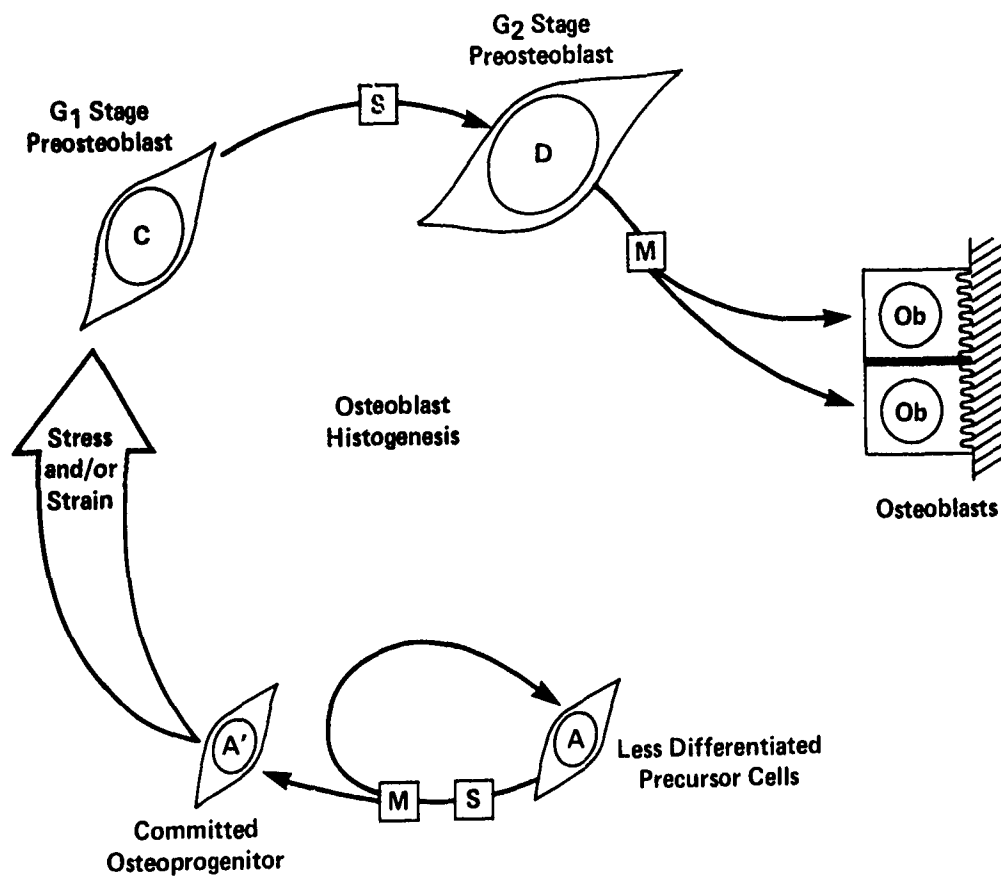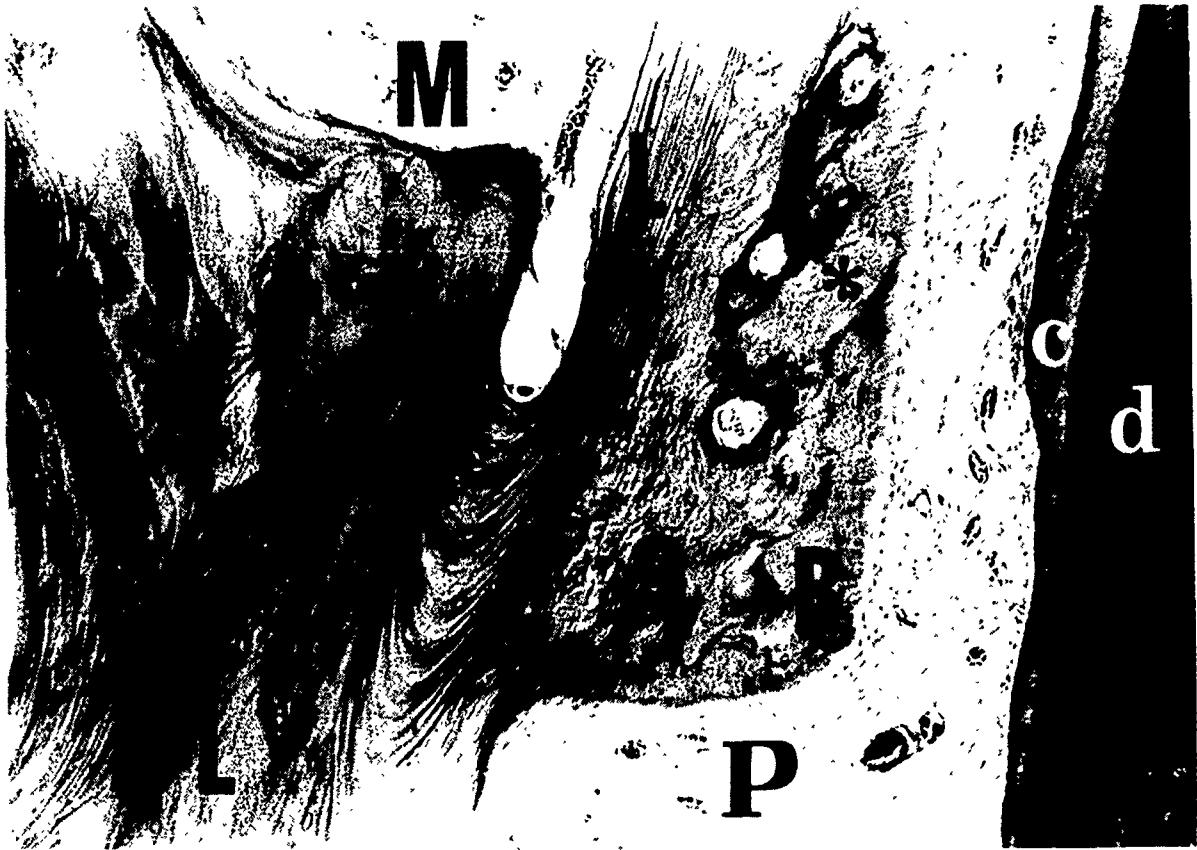
Figure 5
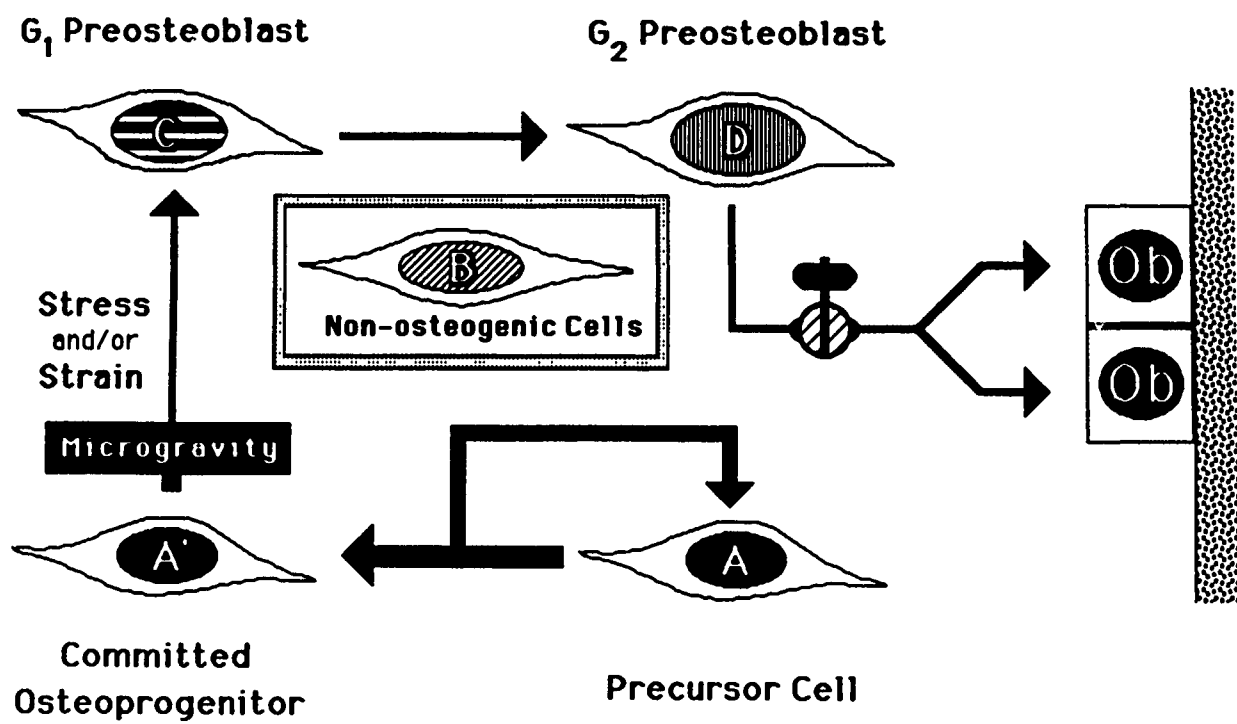
Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

UNLOADED
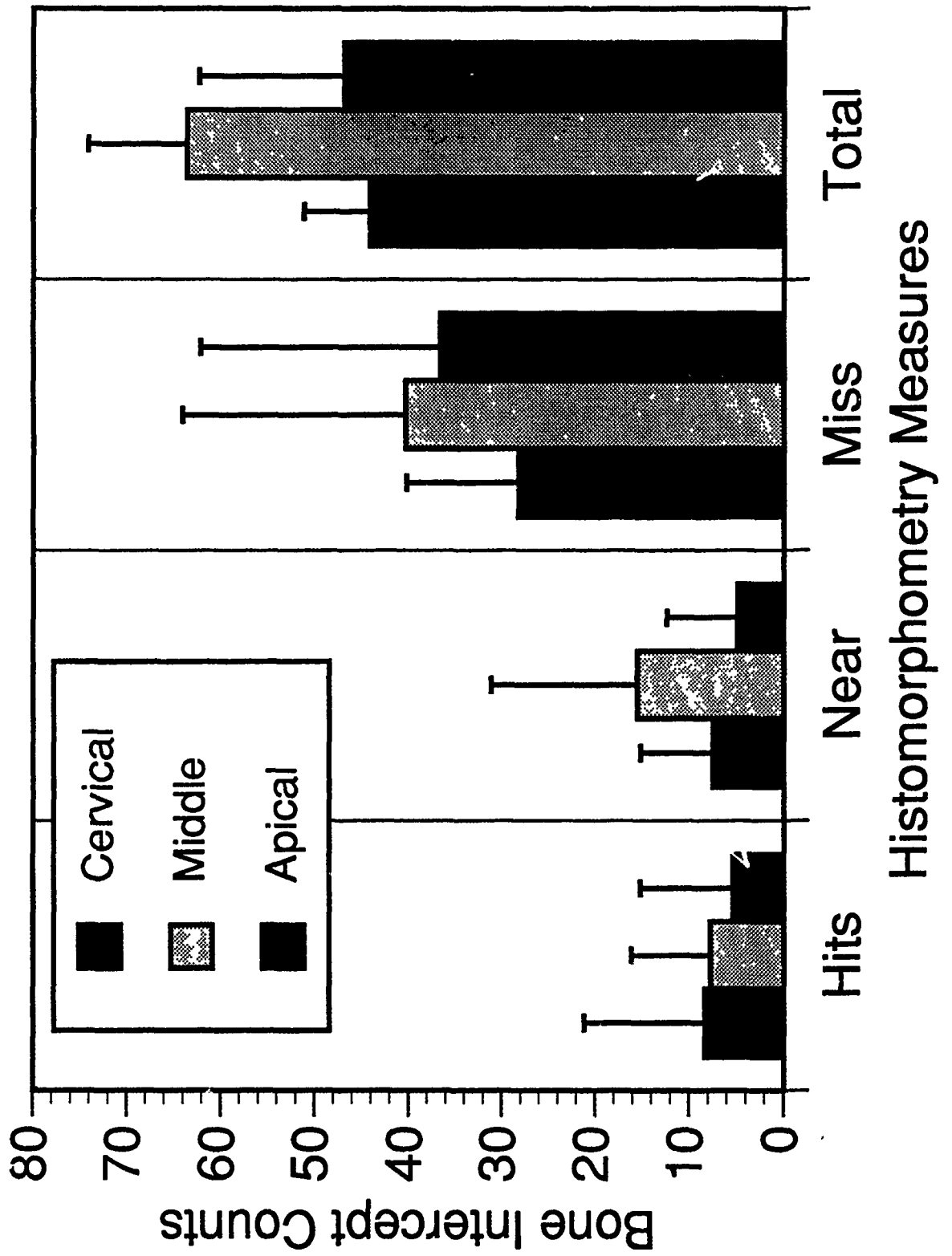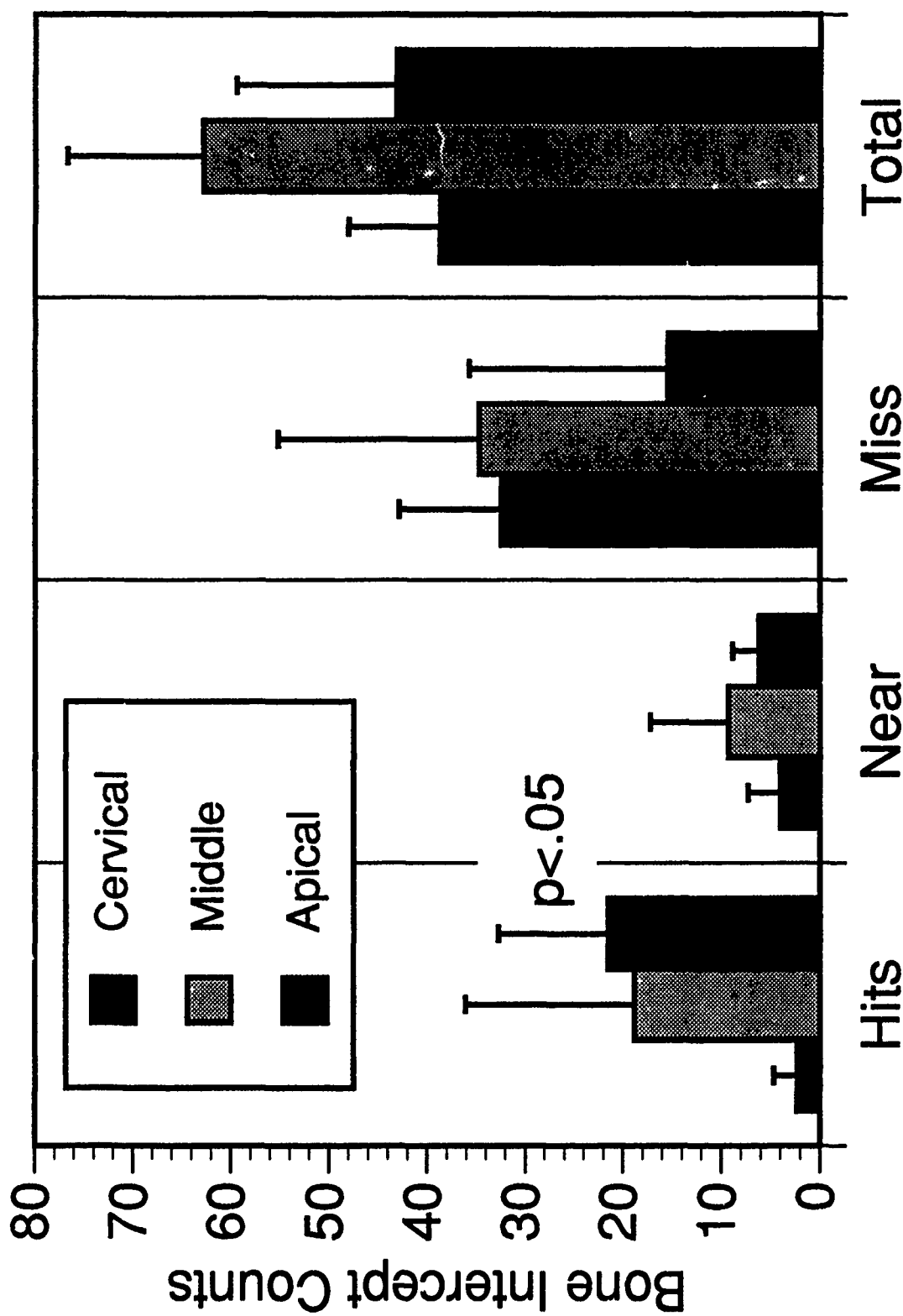
Figure 12

Figure 13

FUNDAMENTALS OF HISTOMETRY FOR BONE GRAFT SUBSTITUTES

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ABSTRACT

All studies of bone graft substitutes at some point include observations of the bone repair response at a histologic level. In this setting, histometry consists of quantitation of bone ingrowth or replacement. With numerical representation of the bone response it then becomes possible to statistically determine the degree of confidence warranted by our observations. It also becomes possible to develop models in which independent variables can be studied as predictors of such response variables as bone ingrowth and implant biodegradation.

The traditional use of histometry in bone research has focused on metabolic bone diseases.[1] Using point counting and perimeter tracing methods, the amount of bone and its surface activity (appositional, resorptive or resting) can be measured. The subjects may be given two doses of tetracycline 10 days apart before sampling the bone. Under UV epi-illumination, the distance between the fluorescent tetracycline bands can be measured and bone appositional rates calculated. Various, metabolic bone diseases can be distinguished by their patterns of bone and osteoid quantity, surface activity, and bone formation rate.

For research in bone graft substitutes, the histologic measures differ in focus from that of metabolic bone diseases. The surgeon wishes to know about union and incorporation of the graft, pore fill by ingrowth of bone into the graft, the gradient (2D) or stereology (3D) of bone ingrowth, and the permanence or biodegradation rates of the graft. Once the process of bone repair has been completed and a "normal" state of bone activity and remodelling is resumed, the traditional methods of bone metabolic - disease histometry again become applicable.

HISTORICAL DEVELOPMENT

In 1842, DeLesse, a French petrologist, measured the composition of geologic specimens by cutting out tin foil to match the polished surface of a rock.[2] After weighing

the foil, he cut out pieces to match the shape of each mineral component visible on the surface of the rock. The collective weight of the tin foil cut-outs for a given mineral component was divided by the original foil eight to yield an areal fraction. DeLesse proposed that this areal fraction was an unbiased estimate of the volume fraction of the rock occupied by that mineral component.

In 1898, Rosiwal, a German petrographer, introduced the use of lineal analysis.[3] He placed a grid of lines over the rock's surface. The length of the line segments overlying each mineral component (intercepts) were measured, summed and divided by the total lineal length of the grid to estimate the area and volume fractions. Since a grid of lines could be placed in the eyepiece of any microscope, this method reduced the capital cost of histometry to that of an ocular reticle.

In 1931, Glagolev, a Russian petrographer, re-introduced the DeLesse principle by using a form of point counting.[4] He recognized that each point on a grid sampled a known area and that the sum of all points categorized as a given mineral component, divided by the total grid points, yielded the same data as DeLesse's tin foil cut-outs. The relative ease of point counting boosted the popularity of histometry.

In 1945, Tomkeieff discovered a relationship between specific surface area and the frequency of lineal intersections of a Rosiwal grid with different components.[5] The surface area, S, of the components contained within a given volume, V, was shown to be inversely proportional to the mean linear intercept, L', where: $S = 2V/L'$.

The total number of intercepts, I, of the grid lines with the component being measured is counted and L' is calculated. If the length of the line is L and it is placed n times on the sample surface, the mean linear intercept will be given by: $L' = nL/I$.

This extended method of lineal analysis is the perferred method of optical histometry for bone graft substitutes because the surface area of the implant and the fraction of this surface covered by bone ingrowth are valuable measures of implant incorporation.

COMPUTER - ASSISTED HISTOMETRY

The advent of inexpensive microcomputers created an oportunity to facilitate the lineal analysis of bone graft substitutes. By substituting the drawing paper and pencil in a camera lucida system with a digitizing pad and puck, the microcomputer was delegated the task of calculating intercepts, intersects, and storing the identity of the

mesurements.[6,7] In practice, the intermediate image seen by the microscopist is a super-imposition of images from three sources: the histology slide, the lineal ocular reticle, and the digitizing puck (seen through the camera lucida). The microscopist moves the puck by hand so that it traverses each grid line. At each tissue boundary the designated puck button is depressed to notify the microcomputer of the boundary identity and its x-y coordinate. From this data, software can calculate: 1)the soft tissue, bone and implant volume fractions; 2) the soft tissue, bone and implant specific surface areas; and 3) the interface surface areas (e.g., surface areas of implant covered by bone versus soft tissue).

While computer-assisted histometry can be practical, two attributes of bone graft substitute specimen preparation cause this method to require substantial time and patience. Since bone ingrowth may not be uniform, it is desirable to perform a stratified random sampling strategy, in which one section from each 1 or 2 mm of specimen length is selected for measurement. This substantially increases the time required for histometric data collection. Additionally, bone graft substitutes are not easily thin-sectioned unless they are decalcified, which usually results in loss of the very bone graft substitute we wish to measure. Generally, thick sections (100 - 200 um) must be first cut with a diamond wafering blade or diamond wire. These can be thinned using diamond lappers. Because the perparation of thin sections is labor intensive, it is often limited to the requirements of qualitative histology and thick sections are used for histometry. These thick sections - because of the problems of projection effect and optical caps - require substantial patience from the microscopist and still lack desirable accuracy and precision.

AUTOMATED COMPUTER HISTOMETRY

To avoid the need for thin-sectioning and observer recognition of histologic components, a histometry system based on backscattered electron (BSE) images from a scanning electron microscope (SEM) has been developed.[8] In this system, the specimens can be of any thickness, up to the limits of the SEM specimen chamber size, the observer fatigue and bias is replaced by computerized recognition of histologic components.

The fraction of an electron beam which is backscattered by a target material (histology section) was determined by Reuter[9] to be a function of atomic number:

$$N = -0.0254 \neq 0.016Z-1.86 \times 10^{-4}Z^2 + 8.3 \times 10^{-7}Z^3$$

N = % of incident electrons which are backscattered

Z = atomic number

By substituting the average atomic numbers for collagen, bone and hydroxyapatite (HA) implant into this formula, it can be calculated that 3.7%, 7.1% and 13.4%, respectively, of the incident electron beam will be backscattered. Thus, when the backscatter signal from a bone graft substitute histology field is collected, digitized and displayed as a frequency histogram, it will have a trimodal distribution that permits software to recognize and analyze the three tissue components (Figure 1).

The mean escape depth of backscattered electrons was determined by Kanya and Okayama[10] to be a function of atomic number, atomic weight and electron energy:

$$Rko = 0.0276AE^{5/3} /Z^{0.889}p$$

Rko = maximum electron range (μm)

E = electron energy (KeV)

A = atomic weight

Z = atomic number

P = density (g/cm^3)

By substituting the values for collagen, bone and hydroxyapartite (HA) implant into this formula, the mean escape depths are respectively 4.1 μm, 3.2 μm, and 2.4 μm. The errors of projection effect and lost caps are thus limited to those of thin sections.

In practice, histologic fields, customarily of 2 x 2 mm size, are imaged with the SEM, digitized, and stored on a microcomputer for subsequent image analysis (Figure 2). The digitized resolution is 256 points per line over 256 lines, for a pixel to pixel distance of 7.8μm. The microcomputer software analyses of the 655,536 points (256 x 256) in less than one minute. For an observer to count this many points manually, it would be necessary to count 1 point per second non-stop for over 18 hours. It also becomes possible to perform histometry on tissue components of small proportion. It can be calculated that with point counts of 65,536 a volume fraction of just 1% can be estimated with a relative standard error 3.8%.

APPLICATION OF HISTOMETRY

The ease and speed of automated histometry permits its application to a variety of studies.[11-14] At its most elemental level, the soft tissue, bone and implant composition of specimens can be readily determined and studied as a function of time, anatomic site, subject, age, blood supply, functional matrix integrity, and implant variable such as chemistry, pore size, and pore interconnections. By subtotaling the image analysis every 0.5 mm, for example, then continuing across multiple adjacent image fields, a gradient of bone ingrowth (e.g., from cortical surface to periosteal surface) can be determined. Since the location of the imaged field is available to the computer, sterologic analysis (e.g., Is there more bone ingrowth in the proximal sections or the distal sections?) can be performed. A similar range of analyses can obviously be performed on the other histometric parameters, such as surface areas and interface areas. Derived values, such as pore fill and surface fraction covered with a specific tissue component can be similarly analyzed.

Although automated histometry of bone graft substitutes presently utilizes the incident imaging of SEM backscattered electrons, the advent of better equipment of thin-sectioning of undecalcified tissues and the introduction of 32-bit operating systems on microcomputers are likely to bring transmitted imaging with tissue recognition based on stain color into reality.

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FIGURE LEGENDS

Figure 1

Frequency histogram of backscattered electron signal from prous hydroxyapatite after skeletal implantation. The left peak represents backscatter from soft tissues and fluid spaced. The middle peak represents bone and the right peak HA matrix backscatter. The mean value for each peak corresponds to the backscatter fraction of 3.7%, 7.1%, and 13.4% respectively for each tissue component.

Figure 2

Digitized image of porous HA implant after skeletal implantation. the HA matrix (white) has become ingrown by bone (grey). Vascular nourishment of the bone is contained within the non-mineralized areas (black).
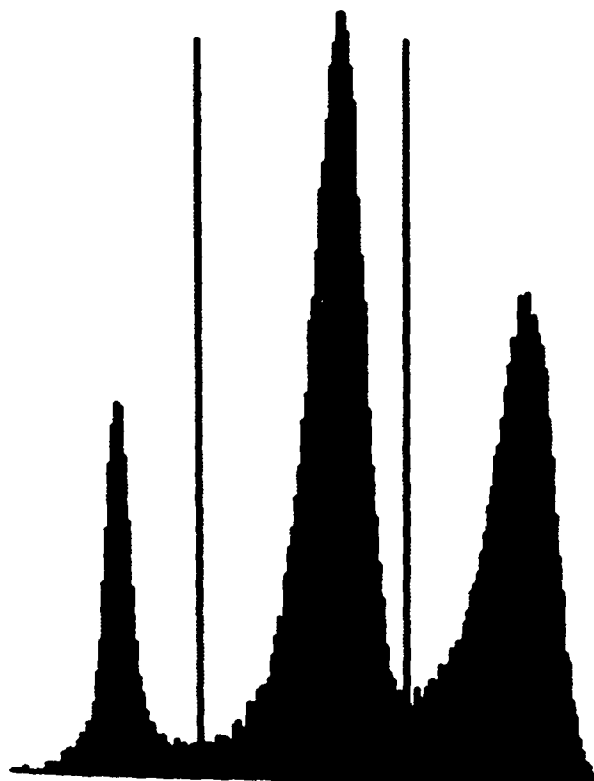
Figure 1

Figure 2

GROWTH FACTORS: AN OPPORTUNITY TO MANIPULATE HEALING AT THE CELLULAR AND MOLECULAR LEVEL

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INTRODUCTION

Surgeons have sought to alter the healing process since the first scalpel was crafted and sharpened. Though centuries have passed, and the surgical manipulation of tissues has been mastered, direct intervention into the healing process has not been successful. With advanced technology, tissues are approximated and sutured with precision, still scar and adhesions continue to form. Clearly, it is the mechanisms that govern the regulation of healing that need further understanding if they are to be manipulated. Evidence suggests that this regulation takes place at the cellular and molecular level, therefore new tools must be crafted. Growth factors are known to regulate cellular function in many processes, including the healing of tissue. By better understanding the specific regulatory activities of growth factors in different tissues, they may soon be used to direct cellular function much as a surgeon now uses a scalpel and suture.

TGF-β, PDGF, and bFGF are among several polypeptide growth factors that are central to the tissue repair processes.[1] These growth factors are released by platelets and activated macrophages, cells that are required for normal wound repair, and appear to act in concert to influence critical activities in inflammation and in the wound healing process.[2,3] For example, PDGF is a potent chemoattractant for wound cells and is able to stimulate critical activities necessary for wound healing within those cells.[4,5] In addition, bFGF has been shown to be a potent mitogenic and chemotactic factor for many cells including vascular smooth muscle cells, preadipocytes, and dermatinocytes.[6] PDGF, bFGF, and TGF-β1 also initiate activities characteristically associated with wound healing when infused into wound chambers implanted subcutaneously in

rats.[7-9] The abilities of PDGF and TGF-β1 to directly accelerate wound healing were first shown in rat incisional wounds.[10,11]

The regulatory role of growth factors in wound repair has included the study of fracture healing. In a fashion that is reminiscent of wound healing, fractured bones heal by a cascade of cellular events in which mesenchymal cells respond to unknown regulators by proliferating, differentiating and synthesizing extracellular matrix. Recent evidence supports a role for growth factors, (PDGF, bFGF and TGF-β1), in the regulation of fracture healing.[16] Both *in vivo* and *in vitro* data suggest that at the initiation of fracture healing, PDGF acts as a stimulator of mesenchymal cell proliferation[8] and stimulates type I collagen mRNA expression. The inhibition of PDGF, with the drug surumin, resulted in decreased cellular proliferation both *in vitro* and *in vivo*, and the inhibition of PDGF during fracture healing resulted in decreased intramembranous bone formation. Therefore, PDGF initiates fracture repair by stimulating mesenchymal cell proliferation and the synthesis of intramembranous bone formation. Basic FGF is a reported mitogen of chondrocytes and osteoblasts,[12] and stimulates cellular proliferation within the fracture callus.[13] In addition, bFGF is a chemoattractant and a stimulant of collagenase secretion in vascular endothelial cells.[14] Basic FGF induces neovascularization by promoting endothelial cell proliferation and capillary cell differentiation.[15] The hallmark of the endochondral ossification stage of fracture repair is angiogenesis and matrix remodeling and mineralization. Therefore, the intense bFGF immunostaining demonstrated in this region[16] suggests a regulatory role for bFGF during this stage of fracture healing. Finally, immunohistologic and *in vitro* explant studies suggest that TGF-β induces the differentiation of mesenchymal cell into osteoblasts and chondrocytes at the early stages of fracture healing.[16]

The mechanisms by which growth factors have promoted wound healing are for the most part unknown. The difficultly is partially the result of the time dependant nature of all healing responses, hence individual activities of specific growth factors may be transitory in nature, or effective only upon cells in transitory stages of differentiation. An extensive literature has evolved on the activities of growth factors in specific experimental situations which does shed some light on their possible function in more complex wound healing. For example, TGF-β has important chemotactic[17] and anabolic[18] actions on fibroblasts involved in tissue repair: it stimulates their production of collagen and fibronectin (as well as their receptors, i.e., integrins),

183

and proteoglycans, and it inhibits the action of proteolytic enzymes[19] which would destroy newly formed connective tissue. The net result of all these actions is the formation of new granulation tissue at the site of action of TGF-β.

To determine the *in vivo* effects of TGF-β and PDGF on skeletal tissue, we developed a model in which exogenous growth factors are delivered into the subperiosteal region of newborn rat femurs. The periosteum is comprised of two tissue layers: an outer fibroblast layer and an inner region of undifferentiated mesenchymal cells.[20,21] Exposure of this population of cells to exogenous growth factors provide an ideal model in which their effects on cell proliferation, differentiation and extracellular matrix synthesis can be studied *in vivo*. Using this model we show that TGF-β, injected subperiosteally into growing bone, initiates a self-propagated tissue response leading to formation of new bone, similar to the response seen in fracture healing. TGF-β alone initiated a cellular cascade of events that included the stimulation of proliferation, differentiation, and extracellular matrix synthesis in target periosteal mesenchymal cells, chondrocytes, and osteoblasts, as occurs during physiological chondrogenesis and osteogenesis. Furthermore, we demonstrated specific differences between TGF-β1, TGF-β2, and PDGF, in their ability to alter cellular activity. This study supports a role for the use of growth factors in the specific manipulation of healing processes.

MATERIAL AND METHODS

In vivo Subperiosteal Growth Factor Injection Model

For injection of TGF-β, a total of 88 newborn Long Evans rats, age 2 to 4 days old, were divided into 6 experimental groups. Animals in groups I, II, III and IV received daily 10 μl injections of 200 ng TGF-β1, 20 ng TGF-β1, 200 ng TGF-β2, or 20 ng TGF-β2, respectively, into the subperiosteum of the anterior, mid-diaphyseal portion of their uninjured right femur for 14 consecutive days; the TGF-β was dissolved in phosphate buffered saline (PBS), pH 7.4. The left femur was injected in a similar fashion with PBS alone, allowing each animal to serve as its own control. Animals were harvested for histology either during the injection phase of the experiment, or at different time points after receiving 14 injections. Animals in group V received seven daily injections of 1% bovine serum albumin (BSA). Animals in group VI received 14 daily injections of 200 ng TGF-β2; on day 15 both legs were used for radiographic and histologic analysis. The

porcine TGF-β1 and TGF-β2 used in this study were purchased from R&D Systems, Minneapolis, MN.

Platelet-derived growth factor used in this study is human B-chain homodimer purified from platelets to over 90% pure as assessed by electrophoresis, and was fully active in bioassay. Exogenous PDGF was injected under the periosteum at the mid-anterior aspect of the femur in newborn LE rats as described above. Either 20ng or 200ng of PDGF was dissolved in 10 μl of PBS and injected into the right leg, while the left leg served as a control and received only a PBS injection. Daily injections were done in triplicate and harvested after 2, 4, 7, and 14 injections for histologic analysis.

Histological Analysis

Tissues were fixed for 2 to 3 days in neutral buffered formalin, two days in Bouin's solution, followed by decalcification in a 10% acetic acid, 0.85% NaCl, 10% formalin solution (AFS).[22] This protocol resulted in adequate decalcification, without loss of antigenic sites for immunohistologic studies. Sagittal and cross sections were obtained in groups I, II, III, and IV, while the specimens in the remaining groups were all sectioned sagittally. Paraffin embedded specimens were stained with hematoxylin and eosin, or Masson's Trichrome (American Histology Labs, Frederick, MD).

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded sections using an avidin-biotin peroxidase detection system (Vector Laboratories), as described by Heine et al.[23] Serial sections of the treated tissue were stained with a polyclonal antibody against type I collagen and a monoclonal antibody against type II collagen[25] after limited hyaluronidase digestion. Sections were counterstained with May Grunwald and Giesma stains. Serial sections developed after omission of either the primary or secondary antibody controlled for non-specific staining. Negative controls consisted of parallel sections incubated with comparable dilutions of rabbit, mouse or goat IgG preimmune serum.

RESULTS

Subperiosteal Injection of TGF-β1, and TGF-β2

Daily subperiosteal injections of either TGF-β1, TGF-β2, or PDGF-BB into the rat femur resulted in a complex and reproducible pattern of tissue formation and differentiation. In contrast, either daily injections of 10 μg/μl BSA or PBS resulted in only minimal periosteal proliferation. Since the most profound tissue response was seen with the 200 ng TGF-β2 dose, this experiment has been used to illustrate the temporal sequence of events induced by injection of TGF-β.

Periosteal Proliferation

Mesenchymal cell proliferation within the inner, cambial layer of the periosteum was seen after two 200 ng injections of TGF-β2, resulting in expansion from its resting, 3 cell layers, to greater than 8 cell layers thickness. The outer fibrous layer of the periosteum remained intact except for mechanical disruption at the injection site. As evidence of proliferation, mitotic figures were seen in a small number of the cells within the enlarging tissue mass. The distribution of responding tissue was specific; the most intense proliferation was at the injection site, with a minimal response laterally, and no effect on the opposing cortex.

Chondrogenesis: Chondrocytes were identified within a cartilaginous matrix after four injections of 200 ng TGF-β2 (Figs. 1). Cartilage, lacking structural organization, formed at the site of injection and enlarged with 7 injections. At this time, chondrocytes appeared immature with a compact cytoplasm and small lacuna. Cartilage not only formed above the cortex, but also appeared to replace underlying cortical bone. Numerous osteoclasts were identified within regions where cortical bone was being resorbed, possibly facilitating the continued invasion of cartilage into the underlying cortex. After 14 injections, the size of the cartilaginous mass had increased several fold and an organizational pattern had emerged. Hypertrophic chondrocytes were seen near the cortical border of the mass, while smaller immature chondrocytes were seen in the vicinity of the proliferating mesenchymal cells nearer the periosteum. Throughout the period of TGF-β injections, the cartilaginous mass was surrounded by areas of intramembranous bone formation.

Intramembranous Bone Formation

New bone was detected in the region lateral to the induced cartilage after four injections of TGF-β2 (Figs. 2). Without evidence for a prior cartilage matrix, it must be assumed that these newly formed bony spicules, lined with osteoblasts, arose directly from the underlying cortical bone by the process of intramembranous bone formation. The mass of intramembranous bone increased in size throughout the 14 days of TGF-β2 injections. Early in this bone formation process, osteoblasts had a rounded appearance, indicative of active matrix synthesis.

To determine the fate of the newly formed cartilage induced following the TGF-β2 injections, animals were sacrificed one and three weeks after TGF-β injections had been stopped. Histologic analysis demonstrated that the cartilage was first replaced with bone by the process of endochondral ossification, and that the bone formed by intramembranous and endochondral ossification was then remodeled into thickened cortical bone.

Endochondral Ossification

The large cartilaginous mass that formed as a result of TGF-β injection underwent endochondral ossification once the TGF-β treatment was discontinued. Histologic analysis demonstrated that chondrocytes developed an organized appearance similar to the growth plate. Columns of these chondrocytes extended perpendicular to the long axis of the bone. Mineralization of the longitudinal septa between columns of cells coincided with chondrocyte hypertrophy. Newly formed endochondral bone had the classic mixed spicule appearance that resulted from the deposition of osteoid (new bone matrix) onto the calcified cartilaginous matrix. Endochondral ossification continued and three weeks after the last TGF-β2 injection (day 35 of the experiment) the cartilaginous mass was completely replaced with bone.

Remodeling

New bone, arising by either endochondral, intramembranous ossification, or existing cortical bone, was continually being remodeled. Histological examination suggested active remodelling as evidenced by the presence of multinucleated osteoclasts resorbing bone within the irregular surfaces of excavation sites along trabecular

bone. This process was followed by the appearance of osteoid-synthesizing osteoblasts, resulting finally in the formation of thickened cortical bone.

A comparison of the effects of TGF-β2 and TGF-β1 on bone formation *in vivo* showed that TGF-ß1 was found to induce the formation of cartilage and bone in a similar, yet not identical, sequence to that just described for TGF-β2. Comparison of TGF–β1 to TGF-β2, at either the 200 ng/day or the 20 ng/day dose, demonstrated a difference in the amount of induced tissue formed. TGF-β2 was found to be more active than TGF-β1, stimulating formation of a mass that was on the average 375% larger at the comparable dose (p < 0.0001, n = 87). Although much larger masses of bone and cartilage were formed following TGF-β2 injections, the ratio of cartilage to intramembranous bone formation within those masses was not significantly different. At the 200 ng/day dose, this ratio was 3.57 for TGF-β1 and 3.71 for TGF-β2 (p = 0.046), while at the 20 ng/day dose, the ratio was 0.0 for TGF-β1 and 0.28 for TGF-β2 (p = 0.049).

The formation of cartilage, at the immediate site of injection, and intramembranous bone, lateral to the site of injection, was a consistent finding. This spatial relationship could be explained by assuming that TGF-β diffused from the injection site with a decreasing concentration gradient, and that the concentration of TGF-β, and not the isoform, determined the pathway of tissue formation into either cartilage or bone. This hypothesis was tested by repeating the injection sequence for both TGF-β1 and TGF-β2 at a lower, 20 ng/day, dose. For both isoforms there was a dose dependent increase in the amount of tissue formed (p < 0.001, n = 87). Whereas, the relative proportion of intramembranous bone formation compared to cartilage formation increased as the dose was lowered. For TGF-β1, decreasing the daily dose from 200 ng to 20 ng decreased cartilage formation from 48.2% to zero (p < 0.001, n = 72) and increased intramembranous bone formation from 13.5% to 47.0% (p < 0.001, n = 72); with TGF-β2, the same dose change decreased cartilage formation from 61.7% to 14.5% (P < 0.001, n = 72) and increased intramembranous bone formation from 16.6% to 51.4% (p < 0.001, n = 72).

In order to further define the composition of the matrix induced by injection of TGF-β2, we stained sections throughout the study with anti-type I and anti-type II collagen antibodies. Anti-type II collagen antibodies selectively stained the matrix surrounding proliferative chondrocytes, but failed to stain the matrix surrounding the hypertrophic chondrocytes (Fig. 3, arrow). Type I collagen was localized throughout

the entire cartilaginous mass; however, staining was most striking around the hypertrophic chondrocytes. In addition, type I collagen antibodies stained osteoid within the newly formed bone spicules, in areas of both intramembranous and endochondral ossification. There was no collagen type II staining in areas of bone formation.

Subperiosteal Injection of PDGF

The subperiosteal injection of PDGF resulted in a dose dependant stimulation of mesenchymal cell proliferation within the cambial layer of the periosteum. After two injections the periosteal thickness had doubled, and with seven injections the periosteum was 8-10 cell layers thick while the control was remained unchanged. In addition, after 7 injections osteoblasts were first detected synthesizing new bone by intramembranous ossification that by 14 injections had increased into a large mass of bone that was now twice the thickness of the opposing cortex. The mass was comprised of intramembranous bone, without evidence of cartilage formation as was demonstrated with the TGF-β injections.

DISCUSSION

In this study, we have summarized previous work in which we found that TGF-β injected subperiosteally into the femur of young rats can initiate a complex series of events resulting ultimately in new bone formation.[16] Our results suggest that not only can TGF-β induce the differentiation of periosteal mesenchymal cells into osteoblasts and chondrocytes but that it can also stimulate these cells to proliferate and synthesize the extracellular matrix proteins characteristic of bone and cartilage. The mechanism by which TGF-β promotes chondrogenesis and osteogenesis is unknown; however, it is likely to involve the possibilities that TGF-β may be chemotactic for cells involved in these processes, or ven less directly, that TGF-β may induce cells to secrete other peptides with these activities.[26,27,3] Moreover, the demonstrated autostimulatory effect of TGF-β injections is certainly an important aspect of its action.

The present study utilized exogenous recombinant TGF-β and PDGF to develop an understanding of how growth factors regulate cellular processes within the periosteum of young rat bones. The study sheds light on the possible role of growth factors in the formation of predicted normal bone and cartilage and also, unexpected fibrous

189

tissue during a simulated repair process. The growth factors used in the present study have already been genetically cloned and are available in a human recombinant formulation in sufficient quantities that they could be used in the treatment of human repair processes. Furthermore, several of these factors are already in clinical trials to determine their efficacy in the treatment of normal and impaired skin repair.[28] In future studies we hope to define specific interventions in the normal regulation of skeletal tissue repair with the goal of improving the final outcome of the repair process in the clinical setting.

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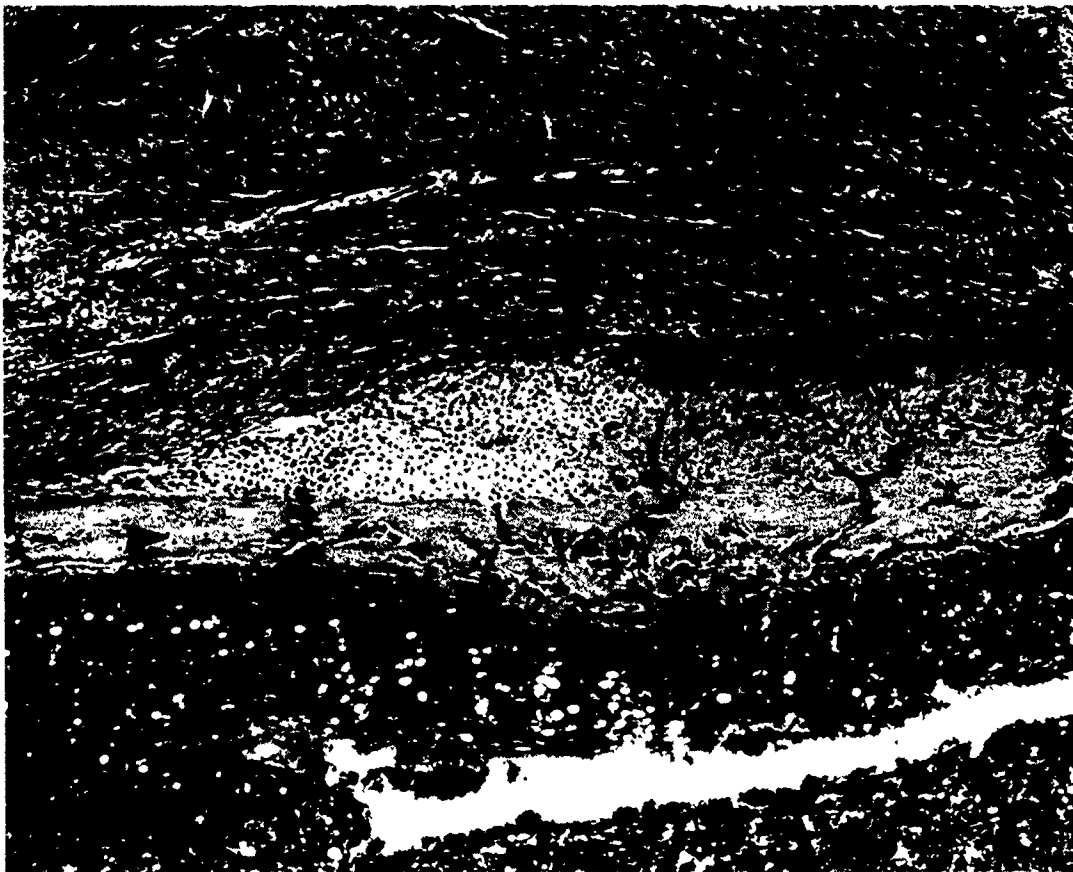
Figure 1

Figure 2
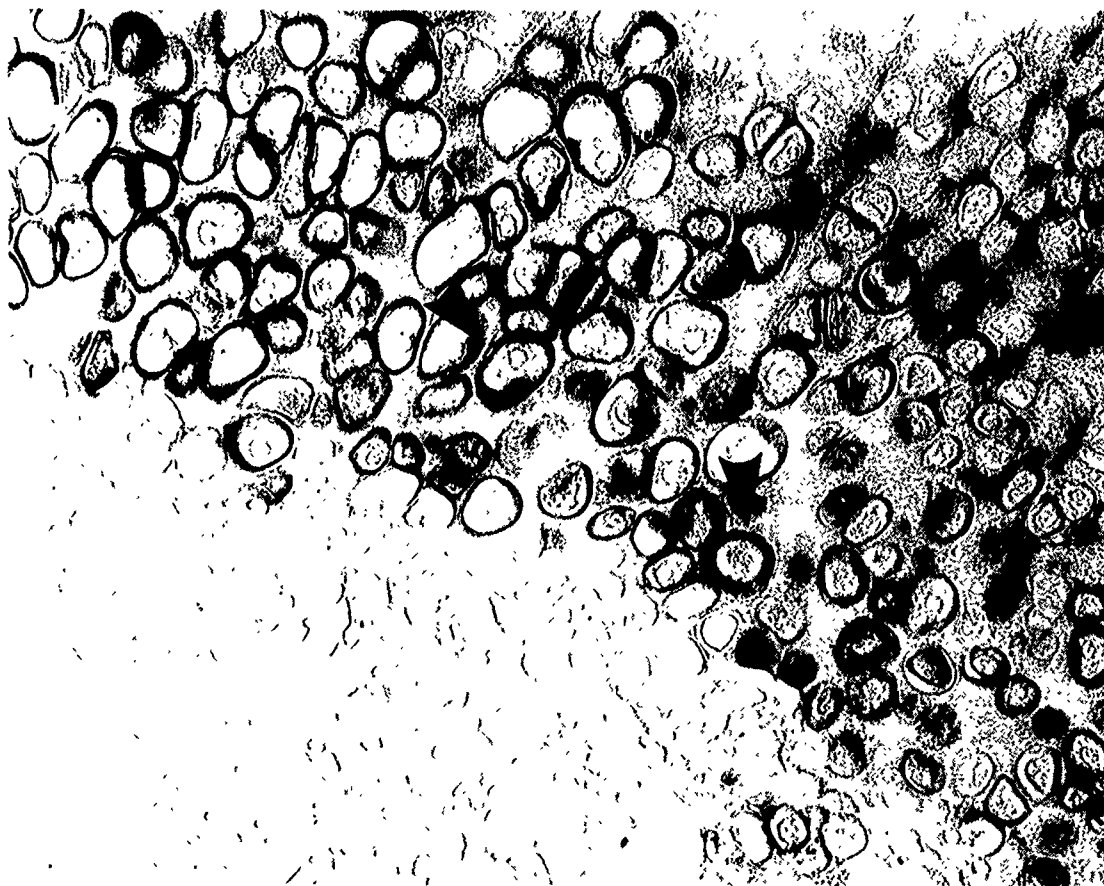
Figure 3

CORRELATION BETWEEN GENE EXPRESSION AND HISTOLOGY SUGGESTS LOCAL REGULATION OF FRACTURE REPAIR

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ABSTRACT

We quantified gene expression in the reparative callus that forms after fracture of the rat femur. Genes analyzed code for extracellular matrix proteins synthesized by proliferating chondrocytes, hypertrophying chondrocytes, osteoblasts, and osteocytes and include alkaline phosphatase, osteonectin, type I and type II procollagen, proteoglycan core protein, and osteocalcin. The reparative callus can be divided into regions of bone and cartilage formation (called hard and soft callus, respectively), and gene expression was examined separately in each region. Expression of extracellular matrix protein genes varied with the progression of repair and differed between hard and soft calluses. Messenger RNAs for osteonectin, alkaline phosphatase, and type I procollagen were detected in the hard callus at maximal levels during endochondral ossification and bone remodeling (day 15), and at 50% maximal levels during intramembranous bone formation (day 7). Messenger RNAs for these proteins in the soft callus were detected at low levels during chondrogenesis (day 9), but increased to 80% of maximal levels with chondrocyte hypertrophy and mineralization of the cartilage matrix (day 13). Messenger RNAs for type II procollagen and proteoglycan core protein were detected at maximal levels in the soft callus during chondrogenesis (day 9). Osteocalcin gene expression was detected in the hard callus during endochondral ossification and remodeling, but not during intramembranous bone formation nor at any time in the soft callus. Osteonectin mRNA was detected in both the hard and soft callus throughout the entire course of fracture repair.

A highly ordered pattern of gene expression was seen as cells in the hard and soft callus regions differentiated into osteoblasts and chondrocytes and synthesized extracellular matrix proteins. Expression of cartilage and bone-related genes correlated

with the appearance of these tissues on histology, suggesting transcriptional regulation of gene expression during repair. Endochondral ossification and intramembranous bone formation correlated with increasing mRNA levels for type I procollagen and alkaline phosphatase. Low levels of osteocalcin mRNA during intramembranous bone formation suggests and absence of osteocalcin in intramembranous bone, and qualitative differences in the bone matrix formed by these two processes. Chondrogenesis correlated with expression of type II procollagen and proteoglycan core protein genes, while chondrocyte hypertrophy correlated with decreased mRNA levels for these proteins and increased mRNA levels for type I procollagen and alkaline phosphatase. These differences in gene expression, both between hard and soft callus regions and in each of these regions over time, suggest local regulation of gene expression by cells in the callus during cell differentiation and matrix synthesis.

INTRODUCTION

Fracture repair is a complicated process which includes intramembranous ossification, chondrogenesis, endochondral ossification, and bone remodeling. The histology of fracture repair has been extensively studied: after injury, mesenchymal cells from the periosteum and surrounding tissues proliferate and differentiate into chondrocytes and osteoblasts. Extracellular matrix proteins synthesized by these cells form reparative tissue, called a callus, external to the fractured bone. Newly formed osseous tissue, referred to as hard callus, initially comprises the peripheral portion of the callus, while cartilagenous and fibrous tissue, called soft callus, is located in the vicinity of the fracture site. With the progression of healing the area of hard callus increases, first as the result of intramembranous ossification then as the result of endochondral ossification, until the entire soft callus is replaced by bone. Remodeling of the hard callus by osteoclasts restores the normal anatomy of the cortical bone (for a review see Brand).

Although the histological progression of fracture repair has been carefully elucidated, little is known about the molecular and cellular events that are responsible for these histological changes. We evaluated mRNA levels for several proteins synthesized in the fracture callus. The mRNAs analyzed in these experiments code for proteins that are essential components of bone and cartilage matrix. Osteonectin, a protein with high affinity for calcium, is felt to be important for the mineralization of

bone and cartilage tissue. Alkaline phosphatase is also important in mineralization, and enzyme activity is detected in mineralizing bone and cartilage matrix. Osteocalcin, a calcium-binding protein, is found only in bone. Proteoglycan core protein, a structural component of cartilage, is important for the incompressibility of that tissue. Type I collagen is the principle protein of bone although it is found in all fibrous tissues, while type II collagen, a cartilage-specific protein, is the principle protein of that tissue. Because these proteins are either tissue-specific or associated with bone and cartilage formation, they serve as markets for cell activity by mature osteoblasts or chondrocytes. Although protein synthesis is under both transcriptional and translational controls, steady-state mRNA levels reflect the amount of protein synthesis. This was illustrated in human fracture calluses by Deutche *et al.* who showed an increase in RNA prior to protein synthesis.[3] We hypothesized that changes in steady-state mRNA levels for these proteins would be indicative of significant alterations in synthetic activity by cells in the callus.

Our aim in this study was to demonstrate some of the molecular events that occur during fracture healing. To accomplish this, the histological changes during fracture repair were documented and gene expression for matrix proteins was analyzed by quantitation of specific mRNA levels in the total RNA extracted from hard and soft callus. Changes in mRNA levels over time correlated with the histological progression of fracture repair, indicating that histological changes were the result of changes in gene expression and subsequent protein synthesis. Messenger RNA levels for specific extracellular proteins varied between different portions of the callus, implying independent regulation of cell function in hard and soft callus.

MATERIALS AND METHODS

Design of the Study

Seventy eight rat femurs were fractured for these experiments. Four fracture calluses were harvested every other day for RNA extractions. Five to eight calluses were evaluated histologically every other day. Messenger RNA levels were quantified by Northern blot analysis as an index of gene expression for extracellular matrix proteins. Histological changes in the callus were compared with mRNA levels for matrix proteins.

Fracture Model

Bilateral femoral fractures in male Long Evans rats (313 ±19 g) were produced as described previously.[6] Sodium pentobarbital, 65 mg/kg body weight, was injected intraperitoneally. Under anesthesia, rats were prepared for surgery by shaving and cleansing both legs. A medial peripateller incision was made and the patella was dislocated laterally, exposing the femoral condyles. A kirshner wire (1.1 mm diameter, 2.7 cm length) was introduced from the intercondylar notch into the intramedullary canal. After closing the knee joints, the mid-diaphysis of the pinned femur was fractured using a special fracture device as described by Bonnarens and Einhorn.[2] The rats were permitted full weight bearing and unrestricted activity after awakening from anesthesia. The rats were sacrificed by CO_2 asphyxiation every other day from day 3 to day 17 after fracture.

Histology

Harvested callus specimens were fixed in 5% formalin, decalcified and embedded in paraffin. Six micron thick sections were cut through the long axis of the femur and stained with Masson trichlome stain (Baker Histolabs, Falls Church, Va).

RNA Extraction and Northern Blotting

The harvested fracture calluses were cleaned from the surrounding soft tissue. Using 2.5X loupe magnification we sharply dissected calluses from fractured femurs into soft and hard tissue. Bone marrow was washed from the femur in the hard callus using PBS containing 0.1% diethylpyrocarbonate (DEPC). To prevent degradation of the RNA, specimens were frozen in liquid nitrogen immediately after harvesting. Total cellular RNA was extracted separately from the soft callus and from the hard callus. Total cellular RNA was prepared as previously describe.[6] Briefly, calluses were homogenized in 4M guanidine hydrochloride containing 1.0% sarcosyl, 67.5mM potassium acetate and 0.1% anti-foam A (Sigma, Co., MO). RNA was pelleted by centrifugation at 32,000 rpm for 18 hours under 5.6 M cesium chloride. The RNA pellet was solubilized in DEPC-treated water, extracted with chloroform, and precipitated overnight with 4 M sodium acetate (pH 5.0) at -20°C. The concentration of RNA was determined by spectrophotomeric absorption at 260 nm. After agarose gel

electrophoresis, RNA was stained by ethidium bromide to determine the integrity of 28S and 18S bands. Ethidium bromide staining also indicated that equal amounts of total RNA was loaded in each lane. Transfer to nylon membrane (Hybond-N™, Amersham) was performed by standard methods.[12] cDNA probes were labeled with ^{32}P by nick translation or random priming kits (Amersham). Northern hybridization with specific cDNA probes was carried out at 42°C for 16 hours in 50% formamide. After hybridization, the filters were washed twice at room temperature in 2x SSC containing 0.05% sodium pyrophosphate and twice at 65°C in 0.1x SSC containing 0.05% sodium pyrophosphate. Autoradiograms were developed after exposure of the nylon membrane to x-ray film for variable periods of time. cDNA probe for pro-alpha1(1) chain of type I collagen was a gift from Dr. D. Rowe, University of Connecticut, Farmington. Alkaline phosphatase probe was a gift of Drs. G. Rodan and M. Thiede from Merck, Sharp and Dohme, West Point, PA. Bone gla protein probe was provided by Dr. V. Rosen, Genetics Institute, Cambridge, MA. cDNA probes for alpha1(II) procollagen and proteoglycan core protein were provided by Y. Yamada and K. Doge, LDBA, NIDR/NIH, respectively.

RESULTS

Histological Change of Fracture Repair

Our histologic findings are summerized in Table 1. The major processes and findings are described below:

1) Intermembranous ossification: The initial responses to fracture included hematoma formation at the fracture site, the initiation of an inflammatory response, and the proliferation of subperiosteal cells. Cells in the inner layer of the periosteum proliferated, increasing from a 1-2 cell thickness prior to fracture (data not shown) to a 6-10 cell thickness by day 3. (Fig. 1A). Newly formed osteoid was also seen at this time on the cortex. As intermembranous ossification proceeded, mature trabecular bone, lined by osteoblasts, began to form and was visible by day 7 (Fig. 1B, C).

2) Chondrogenesis: While subperiosteal cell proliferation and intermembranous ossification were occuring under the periosteum, cells in the soft callus also proliferate, and the size of the soft callus enlarged. At the same time, chondrogenesis was seen in the soft callus adjacent to the newly-formed trabecular bone (Fig 1). By day 5, cells adjacent to trabecular bone had increased in size and synthesized an avascular, ground-

glass appearing matrix (Fig 1B), forming a small cartilage area. The number of cells and size of this avasular area increased. By day 9, cartilage was the predominant tissue in the soft callus, and the majority of chondrocytes appeared to be proliferating (Fig 1D). These chondrocytes began to mature into hypertrophic chondrocytes. On day 11, several types of chondrocytes were present in the soft callus, which resembled a growth plate. Chondrocytes adjacent to the trabecular bone were hypertrophic, while premature and proliferating chondrocytes were found toward the center of the soft callus. On day 13 almost all cells in the soft callus had an appearance typical of hypertrophic chondrocytes.

3) Endochondral ossification and bone remodeling: Osteoclasts were seen in the subperiosteal bone on day 7, indicating that remodeling began immediately after formation of the new trabecular bone. Resorption of cortical bone was not seen until day 9, when Haversian canals containing osteoclasts connected with remodeling spaces in the newly formed subperiosteal trabecular bone. Endochondral ossifiction was first seen on day 9 in hypertrophic cartilage adjacent to trabecular bone. By day 15, endochondral ossification had involved the er "re junction between hard and soft callus (Fig 1G). During endochondral ossification, blood vessels invaded the hypertrophic cartilage matrix. In this region of the hard callus, new bone mineralization formed "mixed spicules" i.e., new trabecular bone with a cartilaginous core covered by mineralized osteoid. Approximately half of the cartilage tissue had been replaced with bone by day 15 (Fig 1G). Endochondral ossification proceeded until bone bridged the fracture site, a process that took between 28 and 32 days in this model (data not shown).

We analyzed a series of fractures to estimate the reproducibility of the histological progression observed during fracture repair in this model. Intermembranous bone formation and remodeling, chondrogenesis, and endochondral ossification appeared at consistent times after fracture (Table 1). Intermembranous ossification started on day 3, and mature trabecular bone was seen by day 7. Cartilage tissue appeared between 3 and 5 days after fracture. Endochondral ossification was seen between days 7 and 9. Remodeling in newly formed trabecular bone and underlying cortical bone started on day 7 and 9, respectively.

Composition of Hard and Soft Calluses

Calluses from fractured femurs were dissected into soft and hard tissue, and were washed free of bone marrow from the medullary cavity (Fig. 2). Then, total RNA was extracted from hard and soft calluses separately. The hard callus was composed of newly formed trabecular bone, underlying cortical bone, and mineralized cartilage. Trabecular bone was formed by both intermembranous and endochondral ossification. The composition of the soft part of the fracture callus depended on the phase of the fracture healing; large numbers of mesenchymal cells on day 3, proliferating chondrocytes on day 9, and hypertrophic cells on day 13.

Quantification of mRNA for Extracellular Matrix Proteins

Total RNA was extracted separately from hard and soft callus. Gene expression for each matrix protein was detected using specific cDNA probes. mRNA message for matrix proteins in the Northern blot analysis altered during fracture repair, and was different between in the hard and soft calluses. Relative mRNA levels were quantified by measuring the density of autoradiograms. The highest level of relative mRNA levels at any time after fracture in either hard or soft callus was set at 100% (data not shown).

Alteration of mRNAs for Extracellular Matrix Proteins During Fracture Repair

mRNAs for several matrix proteins were detected in hard and soft callus. The mRNA for osteonectin, alkaline phosphatase, and type I procollagen were detected by the third day after fracture. On day 5, gene expression reached levels 55, 45, and 10 percent of maximal, respectively, then declined (Fig. 3). Osteocalcin mRNA levels were less than 2% of maximal levels (Fig. 3) during early intermembranous ossification by day 7 after fracture.

During chondrogenesis in the soft callus, mRNAs for several matrix proteins including osteonectin, type II procollagen, proteoglycan core protein, alkaline phosphatase, and type I collagen was detected (Fig. 4). Osteocalcin mRNA was not detected in the soft callus (data not shown). Osteonectin mRNA increased to 50% maximal levels 5 days after fracture, and was seen at the maximal level on day 9. Type II procollagen and proteoglycan core protein mRNAs were detected 7 days after fracture and peaked on day 9 when proliferating chondrocytes were predominant in the soft

callus. Alkaline phosphatase and type I procollagen mRNAs were detected on days 7 and 9, respectively and reached peak levels on day 13 when most chondrocytes were hypertrophic.

While endochondral ossification and bone remodeling were seen on histology, mRNAs for osteonectin, alkaline phosphatase, osteocalcin, and type I procollagen were detected at significant level in the hard callus (Fig. 3). Osteonectin mRNA started to increase on day 9 and peaked on day 11, and remained at a relatively high level until day 15 after the fracture. The expression of alkaline phosphatase, osteocalcin, and type I procollagen also increased by day 9, but peaked later on day 15 (Fig 3).

DISCUSSION

Fracture repair is the result of a complex cascade of cellular events. Because of the high concentration of mineral and extracellular matrix in bone these cellular events are difficult to study. Investigations of fracture repair in the past have focused on histological, biochemical properties of the callus.[8,9,15,16] In an attempt to evaluate regulation of cellular activity during fracture repair more directly, we characterized gene expression using complementary DNA specific for proteins. This investigation showed changing levels of gene expression for extracellular matrix proteins in a well characterized model of fracture repair, and correlated changes in gene expression with cellular events critical to the repair process.

mRNAs for osteonectin, alkaline phosphatase, and type I procollagen are detected in both cartilage and bone tissues. As both chondrogenesis and osteogenesis occur during fracture repair, fracture calluses were separated into soft and hard tissues, and RNA was extracted from each. In addition, bone marrow in the bone canal contains many cells, and we washed the bone canal of the hard c allus to eliminate RNA of bone marrow cells.

Previous studies have shown osteonectin synthesis by active osteoblasts and osteoprogenitor cells.[7] Osteonectin gene expression has been detected in chick epiphyseal cartilage, with highest levels in the resting and proliferating zones.[10] Presumably, osteonectin gene expression detected in the hard callus was from active osteoblasts in trabecular and cortical bone, while osteonectin gene expression in the soft callus was from resting and proliferating chondrocytes. Osteonectin, however, is also

synthesized by other cells, including fibroblasts and endothelial cells during periods of active matrix synthesis.[20] Osteonectin gene expression in the early soft callus could begin in fibroblasts and endothelial cells during granulation tissue formation.

Alkaline phosphatase is known to be involved in the mineralization of both bone and cartilage. This enzyme is present at significant levels in pre-calcified tissue, and intense alkaline phosphatase activity is detected in mineralizing matrix.[17] Immunochemical studies have shown the presence of this enzyme in osteoblasts and in proliferating and hypertrophic chondrocytes.[1] Our study showed that in the hard callus the alkaline phosphatase gene was expressed during the entire fracture repair process. Peak of gene expression on day 15 suggests that mineralization is occuring at the greatest rate at this time. The gene for alkaline phosphatase was expressed in the soft callus later than the osteonectin gene, and peaked on day 13, suggesting that the alkaline phosphatase gene was expressed mainly in hypertrophic chondrocytes prior to cartilage mineralization as well as in bone cells.

Type I collagen is the major protein of bone. Expression of the gene in the hard callus correlated with the formation of bone by osteoblasts similar to osteonectin and alkaline phosphatase. Gene for type I procollagen was expressed in the fracture callus at much higher levels than gene for other proteins. It was because the exposure time on the radiograph in Northern blot analysis was about 1/3 to 1/4 fold of that in detecting other gene expression even though only half of RNA dose was run on the gel. Consequently, relatively low mRNA levels in early fracture repair still represent significant levels of gene expression. In situ hybridization techniques have demonstrated type I collagen mRNA in osteoblasts during intermembranous and endochondral ossification, which is consistent with our results.[13,14] The formation of bone, either by intermembranous ossification or endochondral ossification, is associated with expression of type I procollagen and alkaline phosphatase. Presumably, increasing levels of gene expression represent increasing levels of osteoblast synthetic activity and increasing rates of bone formation. Osteonectin gene is expressed by both osteoblasts and other cells, correlating less with bone formation in this model.

Type I procollagen gene was also detected in the soft callus. Several studies of growth plates have demonstarted immunostaining for type I collagen around hypertrophic cells and in mineralized cartilage.[5,19] In addition, in situ hybridization

experiment has shown type I procollagen mRNA in the cartilage tissue of the fracture callus.[13] In our study, as the proportion of hypertrophic chondrocytes in the soft callus increased, collagen gene expression appeared to shift from type II to type I. Type II collagen gene expression peaked on day 9, then decreased. Gene expression for type I collagen began to increase on day 9, and peaked on day 13. This shift of gene expression from type II to type I procollagen indicates a change in gene regulation as proliferating cartilage changed to hypertrophic cartilage prior to endochondral ossification.

Type II collagen and proteoglycan core protein are cartilage-specific proteins. Increased expression of these two genes correlated with the increase in the proportion of proliferating chondrocytes in the fracture callus. It suggests that these genes were expressed principally in proliferating chondrocytes. Two studies of human and chick growth plates have shown that mRNA for type II collagen is highly expressed in proliferating cartilage zone, and that gene expression is lower in hypertrophic cartilage zone.[10,14] Proteoglycan core protein is synthesized by cells which synthesize type II collagen,[18] indicating that proteoglycan core protein and type II procollagen mRNA is detected in the same type of chondrocytes. In out study, expression of both genes decreased in conjunction with the decrease in proliferating chondrocytes.

Osteocalcin gene expression in our model was limited to the hard callus. This indicates that osteocalcin is bone specific at the level of gene expression as well as protein synthesis. In the hard callus, osteocalcin gene expression was at very low levels, until day 9 after fracture. Gene expression, then, increased rapidly during endochondral ossification and bone remodeling. This suggests that the osteocalcin in not expressed significantly in early intermembranous ossification when non-ossified premature trabeculare structure was forming. This novel observation is also consistent with the studies which indicate that osteocalcin functions during bone mineralization.[11]

The order sequence of gene expression was obserbed in the soft callus that could be correlated with the histological development of cartilage. This sequence began first with expression of the osteonectin gene during early chondrogenesis, followed by the expression of genes for type II procollagen and proteoglycan core protein when the tissue had differentiated into resting and proliferating cartilage. Finally, alkaline phosphatase and type I procollagen gene were expressed when the predominant tissue in the soft callus was hypertrophic cartilage. The variation in gene expression could be caused by the events in fibrous tissue. However, no significant morphological change

or increase of extracellular matrix was seen in the fibrous tissue. Consequently, alteration of gene expression for these extracellular matrix proteins was assumed to be due to cellular changes in the cartilage during fracture repair. We concluded that histologic changes seen in the soft callus during chondrogenesis are the result of changes in gene expression for osteonectin, type II procollagen, and proteoglycan core protein as cartilage matrix is formed.

There was also an ordered sequence of gene activation in the hard callus beginning day 7 after fracture. This began with osteonectin, followed by alkaline phosphatase, osteocalcin, and finally type I procollagen. Expression of all mRNAs reached maximal levels on day 15, when large areas of endochondral ossification and bone remodeling were seen in the fracture callus. Histologic changes during endochondral ossification and bone remodeling seemed to be the result of change in the expression of genes for extracelluar matrix proteins.

These different patterns of gene expression in hard and soft calluses imply independent local regulation of cell function, demonstrating that fracture repair is regulated on a local level. We hypothesize that local regulation occurs, as it does in other tissue, through paracrine and autocrine pathways mediated by growth factors. This hypothesis does not preclude the regulation by circulating factors such as parathyroid hormone, thyroxin, and vitamin D derivatives which are known to have an effect on fracture healing process. Fracture repair is supposed to be regulated co-operatively by local and systematic factors.

Using a novel approach to the study of fracture repair; the evaluation of gene expression, this study analyzed the cellular evens in fracture healing. Understanding these cellular events will increase our knowledge of the regulation of fracture healing, and potentially lead to the development of principles useful in the treatment of pathologic and healing-impaired conditions.

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FIGURE LEGEND

Figure 1 – Histology of rat femoral fracture healing

Rat femurs were harvested 3, 5, 7, 9, 11, 13, 15, and 17 days after fracture (A, B, C, D, F, G, H). Sections were stained with Masson-trichrome. Arrows show the fracture sites. Specific regions were labeled as follows: cortal bone (co), muscle (ms), intramedullary canal (im), periosteum (om), and hematoma (h). Cellular events observed in the fracture callus include: the formation of new trabecular bone (t), and cartilage (c), endochondral ossification (e), and bone remodeling. Bar, 1mm.

Figure 2 – Separation of the fracture callus into the soft and hard callus for RNA extraction

After harvesting the fractured bone, the fracture callus was sharply dissected into soft and hard parts. The bone marrow was washed out from the intramedullary canal.

Figure 3 – Expression of extracellular matrix protein genes in the hard callus

Messenger RNA in the hard callus was detected using cDNA probes for osteonectin, alkaline phosphatase, osteocalcin, and alpha2(I) procollagen. Ethidium bromide staining of the RNA demonstrated equal amount of RNA in each lane. Graphs show relative gene expression in the hard callus during fracture repair.

Figure 4 – Expression of extracellular matrix protein genes in the soft callus

Messenger RNA in the soft callus was detected using cDNA probes for osteonectin, alpha(II) procollagen, proteoglycan core protein, alkaline phosphatase, and alpha2(I) procollagen. Ethidium bromide staining of the RNA demonstrates equal amount of RNA in each lane. Graphs show relative gene expression in the soft callus during fracture repair.

TABLE 1 – APPEARANCE OF HISTOLOGICAL CHANGE IN RAT

Appearance of:	Days after Fracture				
	3	5	7	9	11
osteoid formation under periosteum	8/8*	8/8	–	–	–
carilage tissue	1/8	8/8	6/6	–	–
endochondral ossification	0/8	0/8	2/6	7/7	5/5
osteoclast in trabecular bone	0/8	0/8	6/6	7/7	–

*The ratio of fracture calluses in which each histological change was seen to the number of examined calluses. Five to eight calluses were harvested every other day from day 3 to 11 after fracture, and the appearance of cellular events were investigated histologically. Fracture calluses from day 13 to 17 were also harvested and investigated, and all showed endochondral ossification and osteoclast remodeling of new bone.

Figure 1

Separation of Soft and Hard Callus
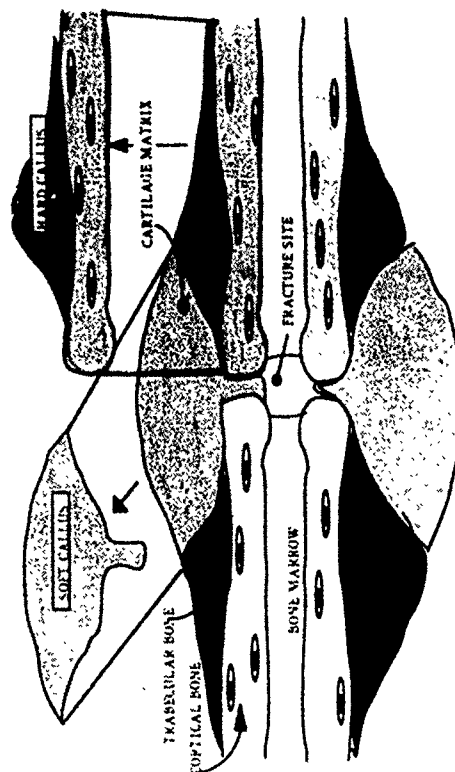
Figure 2
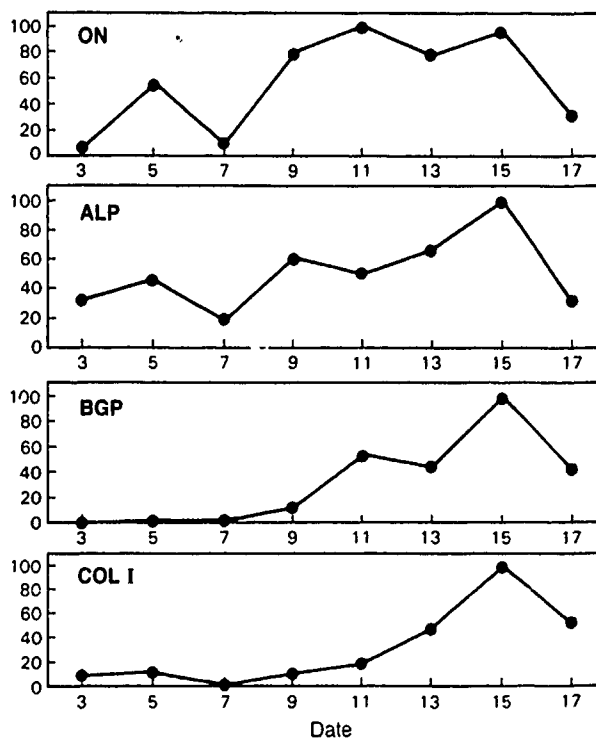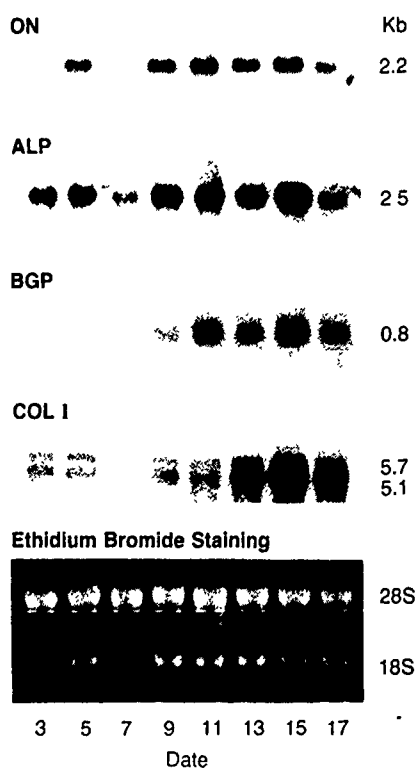
Figure 3
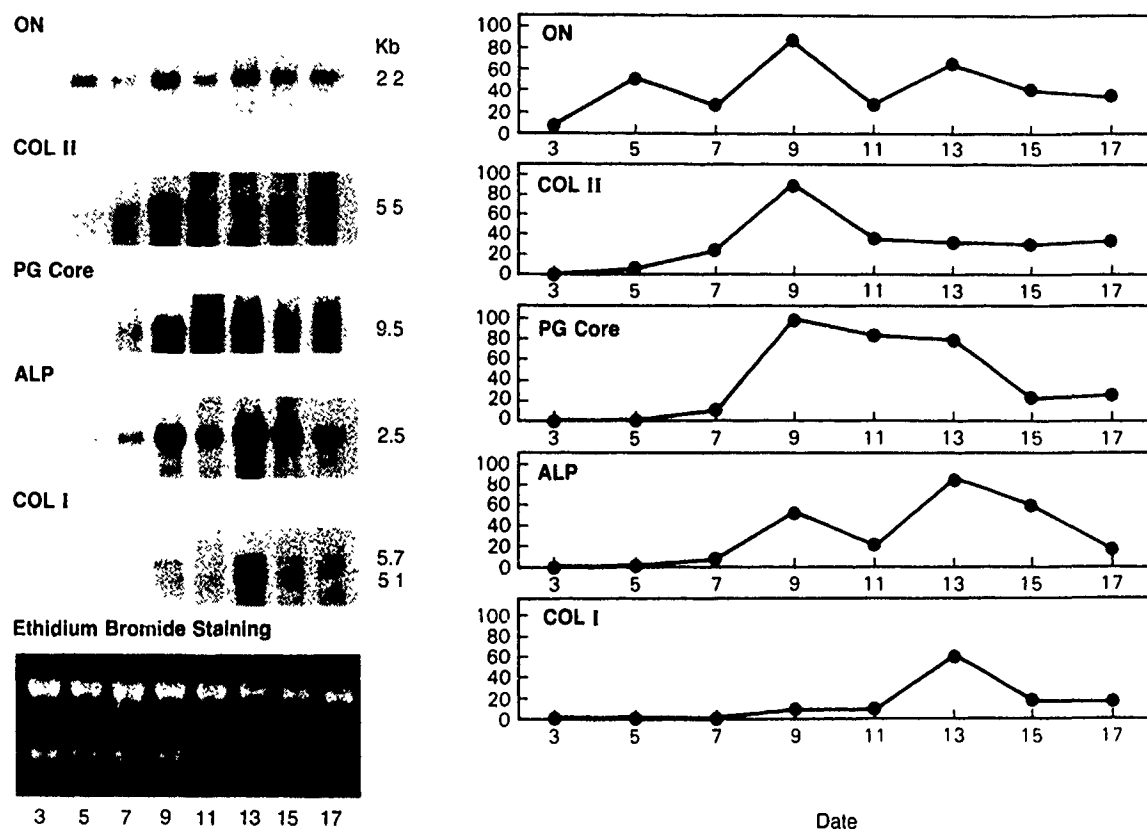
Figure 4

THE TGF-β FAMILY AND BONE REMODELING

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SUMMARY

The TFG-βs are polypeptide growth factors encoded by a family of closely related genes that are expressed in numerous tissues and species. Bone was one of the first tissues in which locally produced molecules with TGF-β-like activity appeared to regulate normal cellular function, and the skeletal matrix probably comprises the largest reservoir of TGF-βs in the organism. *In vitro* and *in vivo* studies indicate that the TGF-β can have stimulatory, inhibitory, and biphasic effects on replication, lineage development, and differentiated function in many types of skeletal tissue cells. Furthermore, a number of other local and systemic factors can regulate TGF-β expression or receptor binding by bone cells, or can increase TGF-β release from the skeletal matrix. Some activities ascribed to the TGF-βs may overlap with those produced by osteoinductive factors and other distally related members of the TGF-β gene family. Consequently, a thorough understanding of TGF-β activity, synthesis, and receptors may results in the rational use of the TGF-βs or related molecules to enhance bone growth or fracture repair.

THE TGF-βs

The TGF-βs acquired their name from the functional assay first used in their characterization and isolation. Cells derived from many tumors spontaneously form colonies in soft agar suspension cultures, while this seldom occurs with cells from normal tissue. Early attempts to isolate tumor-derived products accounting for the neoplastic or transformed phenotype showed that at least two factors were required to support colony formation by an indicator cell line (NRK-49F cells) in soft agar culture,

and the two factors were termed transforming growth factors (TGFs) type α and β. TGF-α shared amino acid sequence, receptor binding, and functional activity with epidermal growth factor (EGF), but had only weak TGF activity. Furthermore, TGF-β by itself was completely ineffective, but together with TGF-α, TGF-β potently induced NRK-49F colony growth.[1,2]

Tissue distribution studies showed infrequent expression of TGF-α, and there was no evidence for its synthesis or storage in bone.[2,3] In contrast, molecules with TGF-β-like activity were found in serum, blood platelets, placenta, kidney, and bone.[1-4] It soon became apparent that TGF-β was similar if not identical to a variety of growth regulators found in several nontransformed tissues, that these molecules had a wide range of activities unrelated to colony formation in soft agar, and that TGF-β-like proteins probably regulated normal tissue function. These proteins included an inhibitor of epithelial cell growth,[2] the activity termed bone-derived growth factor I from fetal rat calvariae,[3,5] and the cartilage inducing factors (CIF-A and CIF-B) isolated from devitalized bovine bone matrix extracts.[6,7] The large abundance of CIF-A and CIF-B (known now as TGF-β1 and TGF-β2) in the bone matrix indicated that skeletal tissue was probably the largest storage site of TGF-β in the organism. Therefore, a close connection between bone and the TGF-βs was evident even in the early stages of these investigations.

There appear to be at least five TGF-βs encoded by distinct but closely related genes. TGF-β1, TGFβ2, and TGF-β3 have been found in many species including humans, TGF-β4 has been found in the chicken, and TGF-β5 has been found in amphibians, and all share about 64-82% amino acid sequence similarity.[8-12] A very high level of sequence homology (greater than 95%) for a single TGF-β isoform among many species argues that these five isoforms are not simply species-specific variants.[12] All five isoforms might be expressed within a single species, in which all or a subset of the TGF-βs might be synthesized by particular tissues, at specific stages of development, or after appropriate stimulation. The active proteins are approximately 25,000 daltons, and are composed of two, usually identical, disulfide-linked subunits each containing 112-114 amino acids. However, a low level of heterodimer comprising one TGF-β1 subunit and one TGF-β2 subunit has been reported in porcine platelets.[9] In most instances, newly synthesized TGF-β is released from the cell in an inactive complex

containing two mature polypeptides, two aminoterminal precursor fragments, and a third protein.[13,14] The third protein is absent in the inactive complex released from a eukaryotic system expressing recombinant TGF-β, suggesting that this component is not necessary for latency.[15] Activation can occur after extreme changes in pH (typically acidification) or with reagents that weaken protein-protein interactions.[13,14,16] The mechanism by which activation occurs *in vivo* is not established completely, but removal of the amino-terminal precursor fragments might involve components of the system used for plasminogen activation.[16] These complexes may be important for protection of nascent TGF-β from proteolysis, and perhaps its maintenance in inactive form during the early phase of the TGF-β life clycle. Other inactive TGF-β complexes containing α2-macroglobulin have also been reported, but these might destine TGF-β for turnover or clearance.[14,17]

CELL SURFACE TGF-β BINDING PROTEINS AND RECEPTORS

In addition to the complexity found in the family of TGF-β proteins, their cell surface binding sites also show a high degree of diversity. Chemical cross-linking studies using cells from a number of tissues demonstrated at least four classes of TGF-β binding sites. Generally, type I TGF-β binding complexes migrate at 65kD on polyacrylamide gels, while those termed types II, III, and IV migrate at 85 kD, >200 kD, and 70-74 kD, respectively.[18]

Type I and type II sites are glycoproteins containing about 5-10 kD of n-linked glycan side chaings.[18] Genetic mutation studies in mink lung cells implicated the type I and type II sites in molecular signal transduction, making them candidates for true receptors.[19,20] In some cells, there is unequal binding at these site for TGF-β1, TGF-β2, and TGF-β3, but this finding does not always translate into differences in the biological effectiveness of the growth factors.[21,22]

Type III TGF-β binding sites are very abundant on many cell types, and bind TGF-β1, TGF-β2, and TGF-β3 with somewhat similar affinity, although this is generally less than the affinities detected at types I and II receptors.[18] The type III sites comprise a moderately complex mixture of high molecular weight proteoglycans that contain core proteins of 100-120 kD, glycosaminoglycan chains of approximately 200 kD, and about 10 kD of N-linked glycan side chains. The type III TGF-β binding stie, recently

termed betaglycan, does not appear to transduce biochemical signals, and may serve as a pericellular site to locate the TGF-βs or related molecules to the surface of cells for storage and perhaps for later activation, or for intercellular communication by way of bound ligand.[19,20,23] Two classes of betaglycan core proteins have been detected. Members of one class contain a presumably small and hydrophobic membrane anchor that localizes the protein to the cell surface, and these molecules can be resolved into at least three molecular weight species after complete de-glycosylation. Members of the second class of betaglycan core proteins lack the membrane anchor sequence and are found in the medium of cultured cells and in serum. Although the proportions of membrane bound to cell-free betaglycan are similar among a number of cell cultures that have been examined, the molecular and biochemical relationships between both classes of core proteins have not yet been fully determined[23] By relation to the other latent TGF-β complexes described earlier, TFG-β in complex with cell-free betaglycan may represent the molecule in mid-life, and also in a protected form, but one from which it may be released for activation of signaling receptors by appropriate, but as yet undetermined, regulatory mechanisms.

Type IV sites are less well characterized. In a pituitary cell culture that does not possess other binding site classes, type IV sites appear to bind TGF-β1, TGF-β2 and the more distal TGF-β-related molecules termed activin and inhibin.[18,24] Osteoblast-enriched cultures from fetal rat bone possess all four binding sites, but preferentially bind the more closely related TGF-β1, TGF-β2, and TGF-β3 at sites other than type IV.[25] Therefore, the type IV sites may actually be the primary receptors for distal members of the TGF-β gene family.

Molecular cloning of the genes encoding any of the TGF-β binding sites has not been reported, precluding direct investigation at the transcriptional level. Nevertheless, several studies, including some with osteoblast-enriched cultures, have demonstrated changes in TGF-β binding during development, aging, or hormonal stimulation.

TGF-βs AND BONE REMODELING

Early studies demonstarted that culture medium conditioned by intact fetal fat bone explants contained biological activity analogous to TGF-β. This activity co-

purified with the material initially termed bone-derived growth factor I.[3] A direct comparison between the biological activities of blood platelet-derived TGF-β and the bone-derived growth factor in calvarial culture assays substantiated that these were closely related if not identical molecules.[26] Subsequently, the bone matrix-derived proteins termed CIF-A and CIF-B were also found to contain similar biological and biochemical features,[6,7] and were probably the bone analogues of TGF-β1 and TGF-β2.[9] Although TGF-β1 and TGF-β2 appear to be the most abundant species in bovine calf bone mmatrix, fetal rat bone cells express at least three TGF-β isoforms (M. Centrella, unpublished results). Furthermore, *in vivo* and *in vitro* studies indicate that TGF-β expression is increased in the fracture callus, and specifically regulates bone-associated activities that may be important for the initiation of fracture repair.[27] These findings provide further strong evidence that TGF-β-like molecules are intimately involved in bone metabolism.

In addition to the closely related isoforms of TGF-β, other molecules with a significant but more d'stal genetic relationship have been found, and are commonly included in the TGF-β gene family. With regard to bone-derived factors, several of these molecules, termed bone morphogenetic proteins or BMPs, share less than 40% gene sequence homology with the TGF-βs.[28] Aside from their osteoinductive potentials,[28,29] there are likely to be some functional distinctions between TGF-βs and the BMPs. No information has accumulated to establish a connection between TGF-β and BMP receptors. Another distal TGF-β-like protein, activin-A, is a homodimer composed of two ß subunits of the heterodimer termed inhibin. Activin and inhibin are both found in gonadal extracts, and counter-regulate follicle stimulating hormone release from the pituitary gland.[24] Activin is similar or identical to the factor termed erythroid differentiation factor, produced in some phorbol-ester treated human monoblastic leukemia cell cultures.[30] Activin has weak TGF-β-like effects in osteoblast-enriched bone cell cultures, but its molar potency is similar to that in other tissue systems. Surprisingly, osteoblast-enriched cultures contain specific activin binding sites that appear to be independent of the primary types I, II and III TGF-β binding sites also found in these cultures.[25] A physiological role for activin in normal bone metabolism is not yet evident, but its over production by some gonadal or monoblastic cell tumors may complicate normal bone remodeling.

Similar to their effects in other tissue systems, the TGF-βs produce pluripotent and biphasic changes in the biochemical activities of cells at particular stages of differentiation within the osteoblast lineage. To date, most studies to evaluate the effectiveness of the TGF-βs in bone have been performed with the prototypical TGF-β1 isoform derived from blood platelets, but TGF-β1, TGF-β2, and TGF-β3 each have identical qualitative effects in osteoblast-enriched cultures from fetal rat bone.[22,31] All three isoforms potently increase DNA synthesis at low concentrations, but have reduced mitogenic activity at higher levels. This could result from interacting signals generated at high and low affinity binding sites. At lower concentrations, signals from high affinity receptors might predominate, whereas at higher concentrations the moderating effects of signals resulting from low affinity binding site occupancy might prevail. Nevertheless, the molecular signals that induce the biochemical effects of the TGF-βs have not been determined in this or any tissue system. All three isoforms also enhance collagen and noncollagen protein synthesis, and decrease alkaline phosphatase activity, but TGF-β3 appears to be 3-10 fold more potent.[22,31] These results correlate well with a higher affinity by TGF-β3 for cell surface receptors in the osteoblast-enriched cultures.[22] Similarly, the TGF-βs enhanced replication and bond matrix production in intact bone explant cultures *in vitro*,[26,32,33] and increase net bone formation in several *in vivo* models.[34,35]

In a variety of continuously cultured nontransformed and osteosarcoma derived cultures, various other effects have been reported. In some human cell systems TGF-β fails to induce cell replication, but enhances alkaline phosphatase activity,[36] whereas in others replication is increased.[37] In certain osteosarcoma cultures, the TGF-βs actually inhibit cell growth.[38] In most cases, however, the TGF-βs enhance bone cell collagen and noncollagen protein synthesis.[22,26,31,33,38-41] Furthermore, TGF-β1 and TGF-β2 potently enhance osteoblast chemotaxis,[42] suggesting an additional role involving cell recruitment that may be necessary for new bone formation or repair.

The stimulatory effect of the TGF-βs on type I collagen synthesis is obviously important in bone structural integrity. Some studies indicate an increase in activation at the genomic level,[31,41] but the extent to which type I mRNA transcripts increase does not appear to account adequately for the total gain in collagen protein that occurs.[31] Recent studies in osteoblast-enriched cultures demonstrate that TGF-β1 increases type I

collagen synthesis in the absence of mRNA transcription, and decreases type I collagen polypeptide proteolysis (Centrella, M., unpublished results). Consequently, the net gain in bone matrix collagen production as a result of TGF-β action probably occurs by multiple, transcriptional, post-transcriptional, and post-translational events.

Additional markers or activities associated with osteoblast function have also been examined. For example, in rat osteosarcoma and nontransformed mouse osteoblast-like cell cultures, TGF-β1 and TGF-β2 each increase the transcript and polypeptide levels of osteopontin, a bone matrix protein throught to be important in cell adhesiveness.[43] The bone matrix protein termed osteonectin, which may be involved in type I collagen deposition and the transition between cartilage and bone, is increased by TGF–β1 in rat osteosarcoma cultures and in an *in vitro* model used for study fracture repair.[27,44] In contract, in rat osteosarcoma cultures TGF-β1 and TGF-β2 decreases transcription of mRNA encoding the bone-sepecific protein osteocalcin, which is believed to be involved in the regulation of matrix calcification.[45]

Other examples of the ways in which TFF-βs alter the expression of structural, functional, and catalytic proteins enriched in bone tissue continue to accumulate. For instance, recent studies indicate that TGF-β1 alone moderately increases prostaglandin E2 (PGE2) synthesis in osteoblast-like cultures from rat and mouse bone, and that interleukin 1 (IL-1) is without effect. However, both molecules synergistically increase synthesis of the prostanoid.[46] This effect may be important with regard to the production of other growth regulators in bone. In this regard, PGE2 potently enhances insulin-like growth factor I (IGF-I) production in osteoblast-enriched cultures,[47] by way of its own stimulatory effect on cyclic AMP synthesis,[48] and IGF-I in turn enhances bone matrix type I collagen production.[49,50] This cascade of interacting events, initiated by TGF-β1, would ultimately enhance bone growth. In other studies, TGF-β1 and IL-1 also synergistically increase the ability of osteoblasts to produce several colony stimulating factors (CSFs) involved in hematological cell development, but effects on CSF production are independent of changes in prostaglandin synthesis.[51]

With regard to cartilage formation, the TGF-βs appear to increase mesenchymal cell differentiation and proteoglycan production, and chondroblast replication.[6,52,53] In contract, these agents usually decrease proliferation and differentiated cell function by more mature chondrocytes. For example, the TGF-βs decrease expression of the type II

and type X cartilage specific collagens, chondrocyte proteoglycan synthesis, and alkaline phosphatase activity.[40,54] Again, biphasic results have been found in some cartilage cell culture models.[53] In general, however, it seems that one important role for the TGF-βs is to induce developmental transitions in cells involved in endochondral bone formation at particular stages of differentiation. The predominant trend of these effects appears to be towards eventual *de novo* bone production.

Much less information is presently available regarding direct effects of the TGB-βs on osteoclasts or osteoclast procursors, due in part to the diffculty in obtaining ample numbers, or sufficiently pure populations of these cells. Early studies indicated that the TGF-β1 could induce bone resorption in mouse calvariae by way of its stimulatory effect on PGE2 synthesis.[55] Other studies then demonstrated that resorption in fetal rat long bones, and osteoclast-like cell formation in human marrow cell cultures, was inhibited by TGF-β treatment.[56-58] More recently, however, biphasic effects on the development of osteoclast-like cells has been reported, in which low TGF-β concentrations were stimulatory , and higher concentrations were inhibitory. In these studies, the stimulatory effects of TGF-β were dependent on prostaglandin production, unifying the apparently conflicting results reported previously.[59]

REGULATION OF TGF-β SYNTHESIS AND ACTIVITY

Several reports suggest that osteotropic hormones and growth factors may influence TGF-β release from the bone matrix, and its synthesis, binding, or activity in bone formation culture models. Preliminary efforts using intact bone organ cultures and fetal rat osteoblast-like cells demonstrated that several hormones known to induce bone resorption, including 1,25 dihydroxyvitamin D3, IL-1, and parathyoid hormone (PTH), each induce TGF-β release to the culture medium, but these studies die not resolve whether the effect were due to the *de novo* TGF-β synthesis, or its release or activation from matrix stores.[60,61] At the transcriptional level, human osteosarcoma cultures treated with estradiol showed increased levels of TGF-β1 mRNA, but polypeptide levels were not determined in these studies.[62] TGF-β1 mRNA has been detected in a number of cell and organ culture models.[39,63,64] In fetal rat parietal bone cell cultures, TGF-β transcripts are relatively stable, and significant changes have only been detected after stimulation with a small number of agents, including TGF-β itself [64] (and Centrella, M., Canalis, E., McCarth T. L., unpublished results).

221

At the receiver level, there are only rare examples of alterations in TGF-β binding. In osteoblast-enriched bone cell cultures from fetal rat bone, the binding capacity at types I, II, and III binding sites is increased by either PTH or the PTH related protein produced by tumors associated with hypercalcemia. The meaning of this finding is unclear, since these hormones reverse most of the biochemical effects of TGF-β.[65, 66] In contrast, glucocorticoid treatment decreases types I and II TGF-β receptors, which are involved in signal transduction, but enhances TGF-β1 binding capacity at cell-associated type III (betaglycan) sites.[67] These effects may indicate that glucocorticoids redistribute TGF-β binding towards non-signaling binding sites, perhaps in part for matrix storage. Decreases in TGF-β binding at signal transducing receptors could be related to one of the ways in which glucocortidoids reduce bone mass and induce an osteoporotic state.

CONCLUSION

Most evidence to date leads to the conclusion that the TGFβs have an important role in bone formation. Research in the field of TGF-β biology in the last six years has clearly included a large degree of effort towards understanding the connection between TGF–β and bone formation. More studies regarding dose, duration, and specificity should help to determine the ways in which these important growth regulators can be used to augment bone growth and to aid in fracture repair.

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TISSUE REPAIR AND GROWTH FACTORS

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INTRODUCTION:
WOUND HEALING AND GROWTH FACTORS

The process of tissue repair following injuries represents a complex series of physiologic, cellular, biochemical and molecular events. These are time–dependent and result in a cascade of redundant biological activities which ultimately lead to a fully healed wound. Soft tissues include the epidermis and dermis, subcutaneous tissue, fascia, muscle, ligament and tendon. The repair processes in these tissues are similar and involve the production of a scar to replace the damaged tissue. The sequence of events in soft tissue repair is also similar to that observed for hard tissue repair, such as bone fracture healing and cartilage repair, although the cells involved in hard tissue repair, i.e., osteoblasts, chondroblasts, possess activities and differentiated functions unique to bone and cartilage, respectively.

Recent identification of probable mediators such as polypeptide growth factors have permitted an increased understanding of the mechanisms and sequence of normal tissue repair. In addition, understanding the differences between normal and abnormal or deficient wound healing is improving through experiments in which the normal tissue repair process is specifically interrupted. Abnormal repair may occur in individuals who receive drugs or radiation or have underlying circulatory or metabolic diseases which affect their capacity to repair wounds, and may reflect defects at the cellular and mediator levels.

Polypeptide growth factors are a class of molecules recently discovered that are responsible for inducing mesenchymally–derived cells such as monocytes and fibroblasts to migrate, proliferate and differentiate. They were initially detected in cellular extracts or conditioned media from normal cells such as platelets, or tumor cell lines,

and were purified based on their abilities to stimulate quiescent target cell lines to proliferate. Once the activity was isolated, the proteins were sequenced, cloned, and expressed to permit more detailed studies of their biological activities.

As a class polypeptide, growth factors are released from 1) platelets, the first cells to enter injured areas; 2) macrophages, which subsequently enter wounds; and 3) fibroblasts, which are responsible for restoring structure to the injured area. Thus, growth factors are secreted and utilized by the cells responsible for repairing wounds. A number of recent preclinical studies performed in animals demonstrate the effectiveness of exogenous growth factor therapy in accelerating normal tissue repair and in reversing the defects observed in compromised tissue repair. These growth factors include platelet–derived growth factor (PDGF), epidermal growth factor (EGF)/transforming growth factor–α (TGF–α), fibroblast growth (FGF), transforming growth factor–β (TGF–β), insulin–like growth factor (IGF), platelet–derived endothelial cell growth factor (PD–ECGF), and keratinocyte growth factor (KGF), a member of the FGF family.

Most growth factors belong to families of related molecules, and each growth factor family has a different mechanism of action and different target–cell specificities, thus they are effective vulnerary agents in different types of soft tissue wounds (Table 1). Each family contains related proteins bearing no homology to proteins within other growth factor families. For example, the FGF family has 8 members, while the TGF–β family has 5 members. Although members within a family may demonstrate some unique functions, they generally interact with cells via the same receptor, triggering identical or similar cellular responses. When growth factors such as PDGF, EGF/TGF–α, insulin–like growth factor II (IGF–II), and basic fibroblast growth factor (bFGF) bind to their receptors, they trigger a receptor tyrosine kinase activity, in which the receptor is autophosphorylated on intracellular tyrosine residues. Other intracellular second messengers are subsequently activated, including G proteins, protein kinase C, and inositol phosphates, which culminate in translocation of the membrane receptor signal to the nucleus and specific early gene, e.g., c–myc, c–fos transcriptional activity. Thus, intracellular signal transduction events initiate molecular and cellular activities considered essential for tissue repair, including cellular chemotaxis, proliferation, and differentiation.

NORMAL SOFT TISSUE REPAIR

Cellular and Extracellular Events

Normal repair occurs in an orderly sequence following injury (Table II). Following coagulation, an acute inflammatory phase results from rapid influx of blood–borne white blood cells including neutrophils and monocytes (which differentiate into wound macrophages) into the wound. These cells are required to clear the wound of bacteria and debris and represent the initial nonspecific immune response to injury. In addition, the macrophages can be activated to secrete a number of growth factors which induce fibroblasts to migrate into the wound. These fibroblasts are also activated by macrophage–derived growth factors to synthesize and secrete additional growth factors and extracellular matrix molecules, such as fibronectin and collagen, structural molecules that are required during the second phase of repair.

The acute inflammatory phase, also called the lag phase, of tissue repair occurs over the first 2–4 days, depending on the size of the wound (Table II). During this time period, neutrophils and monocytes–macrophages predominate in the wound. Wound macrophages are differentiated from circulating monocytes which have been attracted to the wound by various chemotactic factors released during clot formation, including platelet–derived factors such as PDGF and TGF–β, released from platelet alpha granules during degranulation at the wound site. By day 4, fibroblasts from surrounding dermis and underlying connective tissue migrate into the wound and are the predominant cells during the remainder of the repair process. Fibroblast content generally peaks during the first week, then gradually returns to baseline levels over time (i.e., months), as the wound and scar mature. The period of peak fibroblast content within the wound is considered the collagen synthesis phase of repair and lasts from approximately 3 days to 2–4 weeks post–wounding, depending on the size of the wound (Table II). During this time, a high rate of new collagen type I synthesis occurs in soft tissue wounds and accounts for the increased physical strength of the wound. The wound strength increases in linear fashion during the first 10 weeks post–wounding, and is directly correlated with increased net new collagen synthesis.

The final phase of repair, beginning approximately 2–4 weeks after wounding and extending to one year, is the remodeling phase (Table II). During wound remodeling, collagen cross–linking, collagenolysis, and collagen synthesis occur simultaneously,

232

and result in increased structural integrity within the wound. Cross–linking of new collagen is responsible for the significant gains in wound strength achieved during this phase. Collagen breakdown induced by collagenase activity coupled with new collagen synthesis permits increased interwoven collagen bundle formation within the scar. Together, these processes result in a mature collagen-containing scar which is 80–90 percent as strong as unwounded dermis after one year. The processes that stop wound healing once repair is complete have not been identified.

Wound contraction is also an important component of soft tissue repair. Wounds heal by combination of new granulation tissue formation (scar) and contraction of wound edges, thought to be mediated by a specialized cell having characteristics of smooth muscle cells and fibroblasts, the myofibroblast. The myofibroblast appears to be differentiated from wound fibroblasts, contains increased intercellular actin bundles, and is prominent during active wound healing. In loose–skinned animals, wound contraction is critical for survival following wounding. In humans, however, contraction may result in disfigurement and excess scarring (e.g., in burn patients), thus the generation of new tissue to fill an open defect frequently would be a preferable outcome.

Subcellular Events

The three major phases of wound repair offer a useful framework for following the sequence of healing but do not permit one to evaluate the mechanisms responsible for the repair process. Polypeptide growth factors have been recently shown to mediate many functions *in vitro* considered important for wound healing, such as cellular chemotaxis, proliferation, and activation to a differentiated phenotype. The recent availability of purified recombinant DNA–produced growth factors has permitted their systematic evaluation in models of normal and deficient soft tissue repair. Evaluating the influence of polypeptide growth factors on repair processes *in vivo* has permitted the dissection of the cellular and molecular activities required to initiate and sustain a tissue repair cascade. Growth factors may mediate many of the above–mentioned cellular activities considered critical for normal repair of both incisional and excisional (open granulating) wounds (Table III).

Induction of the Acute Inflammatory Phase: Roles of Growth Factors

Monocytes–macrophages are required for normal wound healing. Macrophages synthesize and secrete numerous cytokines and growth factors, including interleukin–1 (IL–1), PDGF, TGF–β, bFGF, and TGF–α, proven vulnerary agents in different models of soft tissue repair (Table III). In addition, growth factors such as PDGF and TGF–β stimulate macrophage chemotaxis and induce the secretion of growth factors such as PDGF and TGF–β. Therefore, through directed cellular recruitment, gene activation, and positive autocrine feedback loops, growth factors such as PDGF mediate macrophage activities required for wound healing. Thus, PDGF augments and exaggerates the acute inflammatory response induced in normal incisional and excisional (open) wound repair. TGF–β is less effective than PDGF in augmenting the acute inflammatory phase because it does not produce the degree of macrophage influx observed with PDGF.

Induction of the Collagen Synthesis Phase: Roles of Growth Factors

Macrophage products such as TGF–β and PDGF are potent chemotactic agents for fibroblasts and may mediate directed fibroblast influx into wounds (Table III). TGF–β and PDGF also activate fibroblast gene expression and proliferation. Activated fibroblast genes include structural proteins such as fibronectin in addition to TGF–β and PDGF. Thus, positive autocrine feedback loops, similar to those identified in macrophages, permit the amplification of an initial exogenous growth factor signal, and may initiate a cascade of activities leading to the increase synthesis of extracellular matrix proteins required for repair (Figure 1).

Both PDGF and TGF–β have profound, but distinct, effects on extracellular matrix (Table IV). PDGF treatment accelerates and augments glycosaminoglycan synthesis within wounds, which establishes the provisional matrix required for cell migration and collagen deposition (Figs. 2 and 3). TGF–β is the only growth factor identified which directly stimulates the transcription of procollagen type I. TGF–β also stabilizes procollagen type I messenger RNA, further increasing synthesis of the protein. Other growth factors, such as PDGF, are considered to increase procollagen type I indirectly, perhaps through the induction of TGF–β (Fig.1). Thus, although both growth factors induce new collagen synthesis within wounds, TGF–β circumvents the acute inflammatory phase, directly increases new collagen formation and accelerates its maturation into mature bundles to a greater extent than PDGF.

In contrast, bFGF treatment of wounds does not appreciably affect the inflammatory phase and actually decreases new collagen synthesis. bFGF induces increased collagenase secretion by endothelial cells, a requirement for vascular invasion and neovessel formation within wounds. bFGF is a potent angiogenic agent *in vivo* and directly stimulates the proliferation and migration of endothelial cells, as well as their differentiation into capillary sprouts, tubes, and functional neovessels (Fig. 4). In contrast, PDGF and TGF-β indirectly induce supporting neovessel formation within wound granulation tissue but do not trigger the marked angiogenic response observed with bFGF. Both PDGF and bFGF, as well as EGF, accelerate re-epithelialization of open wounds, while TGF-β inhibits keratinocyte proliferation. bFGF and EGF are direct keratinocyte mitogens, while PDGF is presumably inducing a keratinocyte mitogen, such as TGF-a or KGF, through its recruitment of macrophages and fibroblasts into the wound. TGF-β also inhibits proliferation of epithelial cell lines in tissue culture.

Wound Remodeling and Collagen Cross-Linking: Roles of Growth Factors

TGF-β decreases collagenase gene transportation *in vitro* and increases transcription of a specific collagenase inhibitor, tissue inhibitor of metalloproteases (TIMP). In contrast, PDGF stimulates collagenase gene expression and therefore may enhance wound remodeling (Table IV). PDGF may have a greater role than TGF-β during the remodeling phase of repair, when more collagenase activity is required to further increase the structural integrity of the wound. Therefore, although both growth factors augment soft tissue repair, they have unique and specific mechanisms of action. Thus, TGF-β stabilizes extracellular matrix while PDGF enhances matrix remodeling; importantly, both growth factors ultimately augment wound integrity without excessive scar formation.

Collagen cross-linking occurs extracellularly via the action of the enzyme lysyl oxidase and is responsible for the majority of wound strength gained after the initiation of the collagen synthesis phase of repair. Growth factors do not appear to influence collagen cross-linking. Thus, PDGF and TGF-β mediate net new collagen synthesis within wounds and influence the amount of new collagen that may be cross-linked, however, they do not influence the subsequent extracellular processing of collagen.

235

Although generation of scar is required in soft tissue repair, the fully differentiated functions of the damaged tissue are restored only in partial thickness injuries (i.e., injuries that penetrate but do not extend trough the dermis). Retained epithelial elements such as glands and hair follicles repopulate the damaged dermis and allow full dermal regeneration. In contrast, in full thickness injuries (i.e., those that extend through the dermis) and injuries involving muscle, tendon, or ligament, the differentiated function of the tissue is not restored, and newly synthesized scar tissue results in a permanent loss of the differentiated cell phenotype and function. Thus, an injury to muscle results in scar formation and not myocyte proliferation and differentiation into a contractile organ. Therefore, the inability to fully regenerate tissues, an activity found in some amphibians, results in scar formation. The mediators that govern full tissue regeneration are unknown but may be similar to those involved in such diverse processes as embryogenesis and scar formation. The inductive potential for full regeneration may be at the level of mediators such as growth factors or at the cellular level (i.e., stem cells) and is presently an area of active investigation.

In summary, exogenous growth factor therapy of experimental wounds accelerates and augments the three processes required for repair, namely re–epithelialization, neovessel formation, and granulation tissue (extracellular matrix) synthesis, through direct and inductive activities (Table V and Figs. 1–4). TGF–β directly increases new collagen synthesis while indirectly enhancing wound neovessel formation. PDGF augments the inflammatory phase, which results in increased release of endogenous growth factors, and inductive effects such as re–epithelialization and angiogenesis (Fig.1). bFGF and EGF both induce accelerated wound re–epithelialization while bFGF also triggers a nearly exclusive angiogenic response within wounds, with minimal synthesis of new collagen.

DEFICIENT SOFT TISSUE REPAIR

In many pathologic states, individuals have comprised wound healing despite the redundant nature of the repair cascade. Drugs such as glucocorticoids and chemotherapeutic agents can adversely affect tissue repair processes. Tissue ischemia, local irradiation, or total body irradiation also diminish tissue repair capacities. Individuals with circulatory diseases such as arterial insufficiency and venous stasis

disease are especially susceptible to chronic non-healing ulcers on their lower extremities. Other individuals with non-healing ulcers include persons with diabetes and neurosensory and neuromuscular defects. Thus, many individuals have compromised tissue repair due to multiple kinds of underlying diseases, although a central pathogenic component involves the sustained loss of viable tissue and repair functions due to inadequate osygenation and nutrition. As understanding the functions of the proteins responsible for normal wound healing increases, proteins such as PDGF and TGF-β will likely be useful therapeutic agents in states of compromised repair. In addition, FGF and PD-ECGF, angiogenic factors that can induce neovessel formation may have a critical role in the repair process, and in the subsequent maintenance of the healed wound.

Macrophages and fibroblasts, but not neutrophils, are required for normal soft tissue repair. The multiple growth factor activities expressed by wound macrophages likely mediate the influx of wound fibroblasts from adjacent tissue and their subsequent activation to synthesize extracellular matrix, including collagen. Thus, an absence or decrease of macrophages of fibroblasts in a wound results in deficient repair. The roles of exogenously applied growth factors in reversing states of deficient repair may increase the understanding of the roles of endogenous growth factors in normal repair.

PDGF and TGF-β have been tested in several animal models of compromised repair (Table VI). Both growth factors are capable of reversing decreased repair, although analysis of their activities in these models have revealed important differences in their mechanisms of action.

Glucocorticoids exert a profound inhibition of tissue repair through induction of a near total monocytopenia resulting in the loww of wound macrophages, and a direct inhibition of collagen synthesis by fibroblasts. TGF-β reverses the glucocorticoid inhibition of incisional healing, likely by substitut8ing for the macrophage requirement of normal tissue repair. In contract, PDGF is not as effective in reversing the deficient repair. In contrast, PDGF is not as effective in reversing the deficient repair. Because PDGF is a powerful wound macrophage chemoattractant and activator of TGF-β synthesis, PDGF appears to require the macrophage to mediate repair.

The macrophage requirement for PDGF activity has been confirmed in studies using local and systemically irradiated rats. Local irradiation prior to incisional wounding kills the dermal fibroblasts required to migrate into the wound and mediate

237

repair. PDGF reverses the resulting healing defect, likely through enhanced chemotactic migration and activation of more distant fibroblasts which were unaffected by the local radiation.

In contrast, in rats receiving total body irradiation to deplete their hematopoietic system, including circulating monocytes, prior to incisional wounding, PDGF does not reverse the deficient repair. Like glucocorticoid-treated rats, the wounds from the total body irradiated rats are characterized by a profound absence of sound macrophages. Thus, despite the inherent redundancy in the repair cascade (Table III), the wound macrophage appears to be critical for normal repair as well as for the acceleration and augmentation of soft tissue repair induced by PDGF.

Tissue ischemia is a central pathogenic element in the induction and maintenance of chronic dermal wounds in patients with multiple underlying diseases, ranging from atherosclerosis to diabetes. Ischemia results in lack of oxygenation and nutrients to the tissue, which is then susceptible to minor trauma. Ischemic wounds d ꞏ ꞏ . ꞏal because the normal phases of repair do not occur at the levels required to sustain a cascade of biological activities due to the lack of oxygen, nutrients, and cellular influx into the wound. The roles of growth factors in antagonizing repair defects of ischemic wounds are only beginning to be investigated.

PDGF reverses an excisional (open) wound repair defect induced after experimentally rendering a rabbit ear ischemic. PDGF-treated wounds demonstrate enhanced extracellular matrix deposition (granulation tissue formation) and supporting neovessel formation. Ischemic wounds treated with the potent angiogenic agent, bGFG, also show enhanced neovessel formation within wound granulation tissue, although the healing rate of bFGF-treated wounds is not as accelerated. bFGF is unable to induce new granulation tissue comparable to PDGF; thus, the local increase in neovessels in the absence of normal alrge vessel flow is not sufficient to reverse dificient repair. Because PDGF does not stimulate endothelial cells directly to undergo differentiation into neovessels, PDGF is likely influencing repair through the enhanced migration and activation of macrophages within the wound, which then release direct angiogenic agents such as bFGF. Thus, the acceleration and augmentation of the acute inflammatory phase of soft tissue repair induced and mediated by PDGF augments normal repair, and reverses the dificient repair process (Fig. 1).

238

REPAIR OF SOFT TISSUES:
POSSIBLE ROLES OF SPECIFIC GROWTH FACTOR THERAPY

In experimental dermal incisional and open granulating (excisional) wounds, acceleration and augmentation of normal repair processes have been observed in wounds treated with PDGF or TGF-β. PDGF augments the cascade of activities observed during the acute inflammatory phase. Both PDGF and TGF-β are potent inducers of enhanced extracellular matrix deposition, an activity responsible for their potent vulnerary effects in normal and compromised tissue repair. Early clinical trials are underway to test the effect of growth factor therapy in accelerating the repair of chronic dermal wounds.

Other types of soft tissue repair processes do not necessarily require the enhanced extracellular matrix deposition and granulation tissue formation induced by PDGF and TGF-β and may benefit from specifically targeted growth factor therapy. These wounds would include partial thickness injuries or vascular surgery, where epithelial–specific (i.e., EGF, bFGF or KGF) or endothelial–specific (i.e., bFGF, PD–ECGF) therapy, respectively, might be more appropriate.

Epithelial Repair

Although PDGF may enhance rates of re–epithelialization via inductive activities, specific keratinocyte proliferation and differentiation factors, such as EGF, TGF–α, FGF, and the recently described KGF may be more appropriate for the treatment of partial thickness wounds (wounds retaining some dermis and epithelial elements such as glands an hair follicles).

Both EGF and bFGF are potent epithelial cell mitogens and have been shown effective in accelerating epithelial repair in such diverse areas as the cornea and the skin. In addition, EGF and TGF–α may stimulate proliferation of mucosal cells lining the gastrointestinal tract (i. e., stomach, mouth mucosa) and the liver and may prove to be clinically useful in treating diseases of the GI tract.

Central and Peripheral Nerve Regeneration

Peripheral neurons, if injured, often result in the permanent loss of innervation to the target organ, due to their minimal capacity of replication and the inflammatory response associated with the injury, which results in scar through which the regener-

ating nerve cannot migrate. Growth factors such as FGF have enhanced the quantity of neurons regenerating after a peripheral nerve resection, and the quality of the transduced signal through the regenerated nerve. Nerve growth factor (NGF) was the first growth factor discovered, more that 30 years ago, due to the serendipitous finding of very high levels in the male mouse submaxillary gland. NGF supports peripheral sensory neurons, and more recently has been found to be active on specific central neuronal populations, such as the basal forebrain cholinergic neurons which are lost in Alzheimer's disease. Like nerve growth factor (NGF), FGF also provides trophic support to the specific types of central and peripheral neurons. Both NGF and FGF may prove clinically useful in neuron repair and regeneration; however, adequate preclinical models of human neurological diseases are not available to test their full treatment potential.

Other newly discovered neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and ciliary neurotrophic factor (CNTF) support the survival and differentiated phenotype of specific neuronal populations. These factors may prove useful in the treatment of neurodegenerative diseases such a Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis; however, much preclinical work must be done first to better characterize the activities of neurotrophic factors. BDNF and NT-3 are members of the NGF family. They are approximately 50 percent homologous to one another, and have overlapping and unique specificities for different neuronal populations. CNTF is unrelated to any other growth factor, and has unique target cell specifities.

Neovessel Formation

The loss of function blood vessels is a major cause of morbidity and mortality in humans. Growth factors such as FGFs, PD-ECGF, and the recently described vascular endothelial growth factor (VEGF), a distant member of the PDGF family, are potent angiogenic agents *in vitro* and *in vivo*. They are capable of stimulating endothelial cell chemotaxis, proliferation, and induction of endothelial sprouts, tube formation, and capillary bud development *in vitro*. Although these activities are considered important for functional neovessel formation, the roles of growth factors in this process remain largely unknown. The mechanisms governing the differential formation of capillaries, veins, or arteries are also unknown.

Functional neovessels would be required clinically in cases of ischemia and would include patients with diseased coronary arteries and diseased large central and peripheral vessels such as the aorta and femoral arteries and in patients with microvascular diseases such as arteriosclerosis of peripheral distal vessels.

Although bFGF is a potent inducer of neovessel formation in experimental granulation wounds, adequate preclinical models with clinical relevance have not yet been described, which would permit a full evaluation of angiogenic growth factors in functional neovessel formation.

Muscle Repair

Wounds in muscle, whether the result of trauma to a skeletal muscle or a myocardial infarction that destroys cardiac muscle, heal via the same processes that govern dermal repair. Thus, muscle injuries heal through cell recruitment, extracellular matrix deposition, and eventual scar formation and not through myocyte–mediated repair.

Growth factors such as FGF are mitogenic for skeletal muscle satellite cells, the precursors of skeletal muscle myoblasts and mature myocytes. PDGF is synthesized by skeletal and smooth muscle cells and stimulates chemotaxis and proliferation in smooth muscle cells. These observations raise the possibility of a PDGF–mediated positive autocrine feedback loop within muscle cells, similar to that described earlier for macrophages and fibroblasts. As with nerve and vessel repair, adequate preclinical models have not evolved to permit a quantitative analysis of growth factor therapy in muscle repair. A model of smooth muscle repair, an incision in the stomach of rabbits, was recently developed and has shown augmented repair with TGF–β therapy. These results, although preliminary, are of importance because most of the gastrointestinal tract is lined with smooth muscle, and inadequate repair following surgery (i.e., intestinal resections) is a major source of morbidity and mortality in patients.

Tendon and Ligament Repair

Tendon and ligaments are modified collagen–containing soft tissue structures that have tensile and elastic properties. When injured, as in the case of injured muscle, scar tissue forms, which frequently may inhibit or diminish the function of the tendon or

241

ligament. An adequate inducer of repair that does not stimulate inflammation and subsequent scar formation has not yet been identified

Relationship of Soft Tissue and Hard Tissue Repair Processes

Many similarities in the sequence of soft and hard tissue repair have been observed. An inflammatory phase and collagen synthesis phase are common to both types of repair; however, cartilage and bone repair requires differentiated functions of chondroblasts and osteoblasts, respectively.

Cartilage Repair

Cartilage, an avascular organ, is repaired slowly through proliferation of chondroblasts and subsequent synthesis and deposition of collagen type II. Growth factors such as FGF, TGF-β, IGF, and PDGF have been shown to stimulate chondroblast proliferation *in vitro*. Although FGF has also been shown to enhance articular cartilage repair, development of reproducible and quantifiable models of cartilage injury and repair have proved difficult.

Bone Repair

Fracture repair is characterized by an initial inflammatory cell influx, proliferation of osteoblasts, their differentiation into collagen type I–producing cells, and subsequent calcification of the matrix. Growth factors have been implicated in fracture repair.

Bone represents the largest source of TGF-β in the body. In addition, bone contains significant quantities of bFGF and a group of osteoinductive proteins collectively known as bone morphogenetic proteins (BMPs). Several have been cloned and are either related to the TGF-β family or known as osteogenin. They share the ability to induce mesenchymal stem cells to differentiate into chondroblasts and osteoblasts, hence the term osteoinductive agents.

Quantitative and reproducible models of fracture healing have been difficult to establish. *In vitro* multiple growth factors, including IGF, FGF, EGF, TGF-α, TGF-β, BMPs, and PDGF have been shown to influence proliferation and differentiation of osteoblasts; however, the relevance to *in vivo* fracture healing remains speculative.

RELATIONSHIPS BETWEEN TISSUE REPAIR, DEVELOPMENT, AND OTHER HYSIOLOGIC AND PATHOLOGIC PROCESSES

In many respects, the cells and activities required for tissue repair resemble the processes that occur in such diverse circumstances as embryogenesis, oncogenesis, metastasis, atherosclerosis, and inflammatory diseases such as rheumatoid arthritis and pulmonary fibrosis. Growth factors released from mesenchymal cells are considered to play critical roles in inducing and sustaining some pathologic activities. Inflammation outside of an appropriate setting such as tissue repair involves recruited and activated macrophages and fibroblasts and likely results in a similar cascade of redundant growth factor and extracellular matrix activities, as observed in wound healing (Fig. 1). In pathologic states, components of this cascade may lead to inappropriate cellular proliferation, fibrosis, granulation tissue formation, and scarring, resulting in such diverse diseases as intimal hyperplasia (i.e., an atherosclerotic plaque), restrictive pulmonary fibrosis, synovitis, liver cirrhosis, and other chronic inflammatory diseases.

Increased levels of PDGF have been observed in the placenta and uterus during pregnancy, suggesting an autocrine physiologic role for PDGF in trophoblast and smooth muscle cell growth, respectively. Other growth factors, such as bFGF and IGF–II have also been found in the placenta. These growth factors may gain access to the fetoplacental circulation and could potentially influence fetal growth and differentiation.

Importantly, growth factors such as FGF, TGF–β, and PDGF have been implicated in critical mesoderm inductive processes in early morphogenesis. They are maternally encoded and therefore are present within the egg at the time of fertilization. Their critical roles in embryonic stem cell migration and differentiation result in the absence of detectable genetic growth factor deficiencies; such deficiencies likely would be lethal. Later in pregnancy, growth factors such as bFGF and TGF–β have been localized to discrete cell populations at specific times of organ or tissue growth. Both growth factors have been found in diverse mesodermal tissues; however, bFGF also has been localized to basement membranes, where it is thought to be released in the adult during tissue repair. Establishment of specific growth factor mediated functions during development,

such as organogenesis, is not possible with these descriptive localization studies. Functional correlates may be possible in the future using transgenic mouse models in which specific growth factor deficiency states have been engineered.

Thus, synthesis and secretion of growth factors under appropriate circumstances likely permit critical homeostatic functions such as wound healing, placental and uterine growth, and embryonic differentiation to occur, whereas aberrant control of growth factor synthesis and inappropriate secretion may result in fibroproliferative diseases or tumor growth and metastasis.

CONCLUSION

Significant and rapid progress has been made in dissecting and elucidating the complex redundant sequence of soft tissue wound healing. However, the exact roles of growth factors in mediating this process require considerably more investigation before rational targeted intervention can be achieved. Growth factor–mediated *in vitro* activities are not necessarily replicated within the complex *in vivo* milieu, where other cells and growth factors may either mask or inhibit activities or enhance inductive effects. Recent availability of recombinant proteins has permitted initial clinical evaluations of growth factor therapy to proceed in people with deficient tissue repair. Only in identifying specific time and cell–dependent roles of individual growth factors in the repair process can appropriate theraputic use of growth factors, either alone or in combination, occur. Thus, further preclinical and clinical *in vivo* investigations are required to identify specific influences of growth factors on each phase of repair, and their influence on the induction of endogenous growth factors and extracellular matrix molecules required to sustain the repair cascade. Future experiments likely will further identify the unique roles of specific growth factors in tissue repair, development, normal physiologic processes, and inflammation and malignancy. The precise regulation of growth factor gene expression within tissues may represent the critical difference between states of normal and uncontrolled cell growth. Thus, tissue repair models represent a useful paradigm in which to explore the diverse biological activities of growth factors.

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FIGURE LEGEND

Figure 1

Model of PDGF and TGF-β mediated soft tissue repair. PDGF augments the inflammatory phase of repair, resulting in an enhanced cascade of endogenous wound growth factor activities, in turn leading to increased deposition of new extracellular matrix and a healed wound. TGF-β, in contrast, circumvents the acute inflammatory phase, and acts directly at the level of the wound fibroblast to trigger matrix deposition (granulation tissue formation) within the wound.

Figure 2

PDGF–enhanced incisional wound repair. PDGF was applied to one (a) of two paired incisions at the time of surgery; the other incision (b) served as a control. Five days later, the incisions were analyzed: the PDGF–treated wound had markedly increased deposition of new extracellular matrix (arrows, scar). D, dermis; E, epidermis. 100 times.

Figure 3

PDGF enhanced open wound repair. PDGF was applied to one full thickness open wound (a) on the rabbit ear; the other wound (b) served as an untreated control. After 10 days, the wounds were analyzed for glycosaminoglycan deposition using an Alcian blue stain. PDGF–treated wounds had markedly increased cellular influx and extracellular matrix deposition (arrow, leading edge of new tissue deposition). Ca, cartilage base of wound; E, epidermis. 40 times.

Figure 4

Basic FGF enhanced neovessel formation. bFGF was applied to one full thickness wound (a) on the rabbit ear; the other wound (b) served as an untreated control. After 7 days, endothelial cells forming new capillaries were immunohistochemically identified (brown stain) in wound sections. bFGF–treated wounds consisted almost entirely of activated endothelial cells and neovessels. Higher magnification (c) reveals proliferation endothelial cells differentiating into capillaries. Some capillaries are functional (i.e.,

contain blood) while others are more immature and are not yet connected to the vascular system. a, b, 100 times; c, 400 times.

TABLE I – MAJOR GROWTH FACTOR FAMILIES IMPORTANT IN SOFT TISSUE REPAIR

Growth Factor Family*	Major Sites of Synthesis	Target Cells(s)	Activities
PDGF	platelets, macrophages, fibroblasts, endothelial cells, smooth and skeletal muscle cells	neutrophils, macrophages, fibroblasts, smooth muscle cells	chemotaxis gene activation proliferation
TGF-β	nearly all cells, largest sources are platelets, bone	nearly all cells	regulates proliferation and differentation of multiple cell types
TGF-α/EGF	platelets, macrophages, keratinocytes	epithelial cells, endothelial cells, fibroblasts	proliferation gene activation
FGF	macrophages, fibroblasts, endothelial cells, central nervous system	endothelial cells, epithelial cells, fibroblasts, chondroblasts	proliferation chemotaxis gene activation capillary formation
IGF	liver, mesenchymal cells, central nervous system	nearly all cells	proliferation, metabolic control
KGF	fibroblasts	epithelial cells	proliferation
PD-ECGF	platelets, placenta	endothelial cells	proliferation chemotaxis capillary formation

*PGDF, platelet-derived growth factor; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; KGF, keratinocyte growth factor

TABLE II – CASCADE OF BIOLOGICAL ACTIVITIES
IN SOFT TISSUE REPAIR

Phase	Time	Cell Influx	Biological Activities
Coagulation	immediate	platelets	initial growth factor release
Actute inflammatory	day 0 to day 4	neutrophils monocytes-macrophages	phagocytosis, protease secretion phagocytosis, growth factor secreation
Collagen synthesis	day 3 to day 30	fibroblasts endothelial cells, epithelial cells	growth factor secretion, glycosaminoglycans, fibronectin and collagen synthesis growth factor secretion angiogenesis reepithelialization
Remodeling	day 14 to 1 year	no influx fibroblasts present	collagenase-mediated remodeling, collagen synthesis, collagen cross-linking
Cessation of Repair	from 1 year	no influx	unknown mediators/inhibitors

TABLE III – WOUND HEALING CELL TYPES
AND ASSOCIATED GROWTH FACTORS

Cell Type	Growth Factors Secreted	Probable Growth Factor Medicated Activities
Platelets	PDGF	Neutrophil, monocyte, fibroblast chemotaxis and activation; fibroblast proliferation
	TGF-β	Fibroblast chemotaxis, activation
	EGF	Keratinocyte chemotaxis, activation, proliferation
	PD-ECGF	Endothelial cell chemotaxis, activation, proliferation, differentiation
Neutrophils	None detected	None identified
Monocytes/ Macrophages	PDGF	Augment, sustain acute inflammatory phase; recruit fibroblasts
	TGF-β	Fibroblast extracellular matrix synthesis
	TGF-α	Same as EGF
	bFGF	Similar to PD-ECGF; neovessel formation
	IL-1	Induces PDGF synthesis in fibroblasts
Endothelial cells	PDGF	As above
	bFGF	Autocrine stimulation
Keratinocytes	TGF-α	Autocrine stimulation
	TGF-β	Possible inhibition of keratinocyte proliferation
Fibroblasts	PDGF	Autocine stimulation, collagenase secretion Extracellular matrix synthesis
	TGF-β	Collagen synthesis
	KGF	Keratinocyte proliferation

TABLE IV – INFLUENCE OF GROWTH FACTORS
ON WOUND EXTRACELLULAR MATRIX

PDGF: ↑   Collagenase

                ↑   Glycosaminoglycans

                ↑   Procollagen type I

                =   ↑ Matrix Remodeling

TGF-β   ↑   Procollagen type I

                ↓   Collagenase

                ↑   Collagenase inhibitor (TIMP)

                ↑   Plasminogen activator inhibitor

                =   Stabilized Matrix

FGF: ↑   Proteases

                =   Endothelial cell sprouts and buds,
                       neovessels

TABLE V – VULNERARY INFLUENCE OF GROWTH FACTORS ON THE MAJOR BIOLOGICAL PROCESSES REQUIRED FOR SOFT TISSUE REPAIR*

Growth Factor	Reepithelialization	Neovessel Formation	Granulation Tissue
PDGF	+	+	+
TGF-β	0/-	+	+
EGF/TGF-α	+	0	0
IGF	0	0	0
FGF	+	+	0/+

*+, positive effect
0/-, no or slight inhibitory effect
0/+, no or slight positive effect
0, no effect

TABLE VI – ROLES OF PDGF AND TGF-β IN REVERSING HEALING DEFICITS IN IMPAIRED TISSUE REPAIR MODELS*

Model	Biological Deficiency	Therapeutic Effect
Glucocorticoid treatment	Macrophages, collagen synthesis	TGF-β
Total body irradiation	Macrophages	TGF-β
Local irradiation	Fibroblasts, collagen synthesis	PDGF
Streptozotocin-induced diabetes	Metabolic	PDGF
db/db diabetic mouse	Metabolic, obesity	PDGF, bFGF
Chemotherapy	Fibroblasts, collagen synthesis	TGF-β
Arterial insufficiency	Blood-borne nutrients, mediators, cells	PDGF

*Reviewed in Cromack, Pierce, and Mustoe (1991)

MECHANISM OF ACTION OF PDGF AND TGF-β IN DERMAL WOUNDS
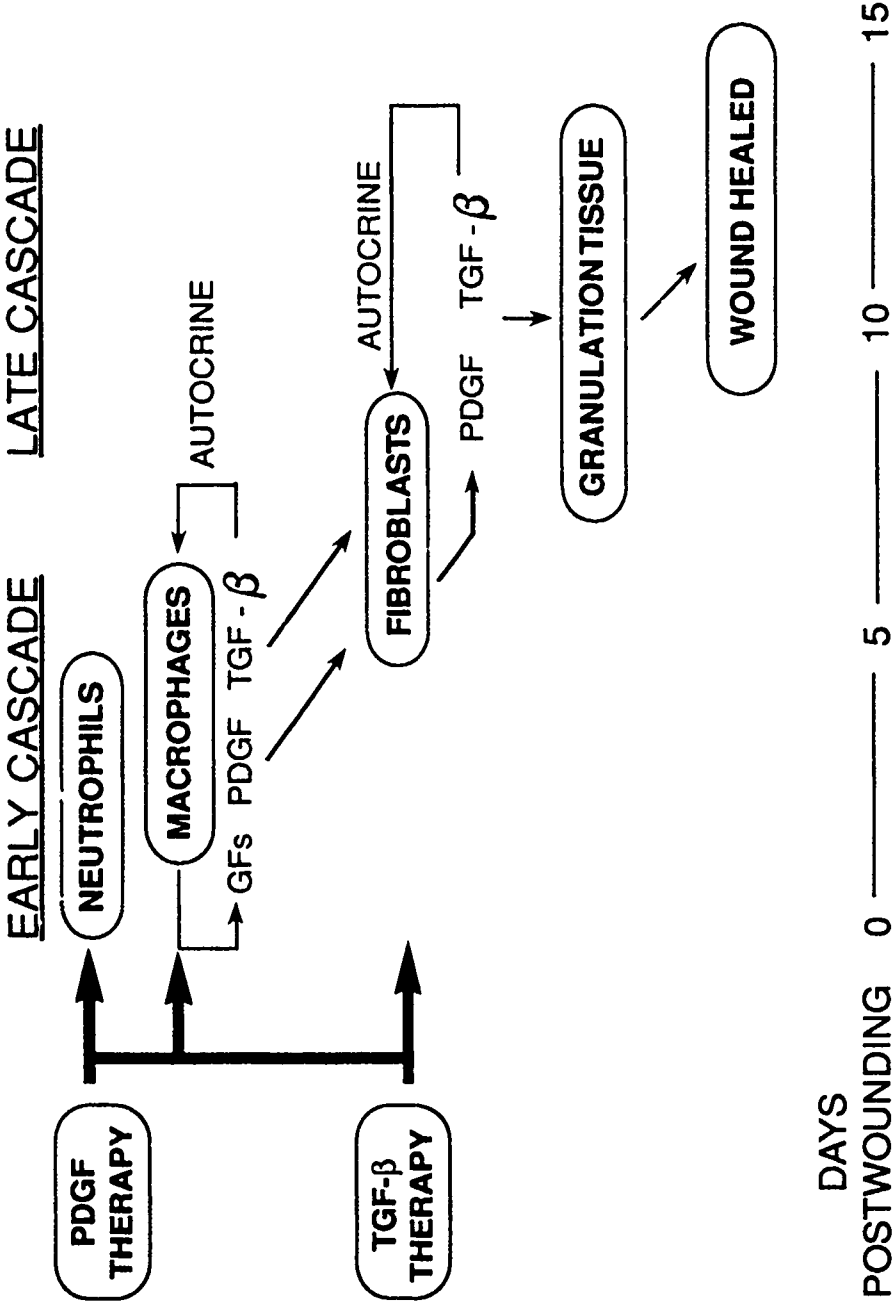
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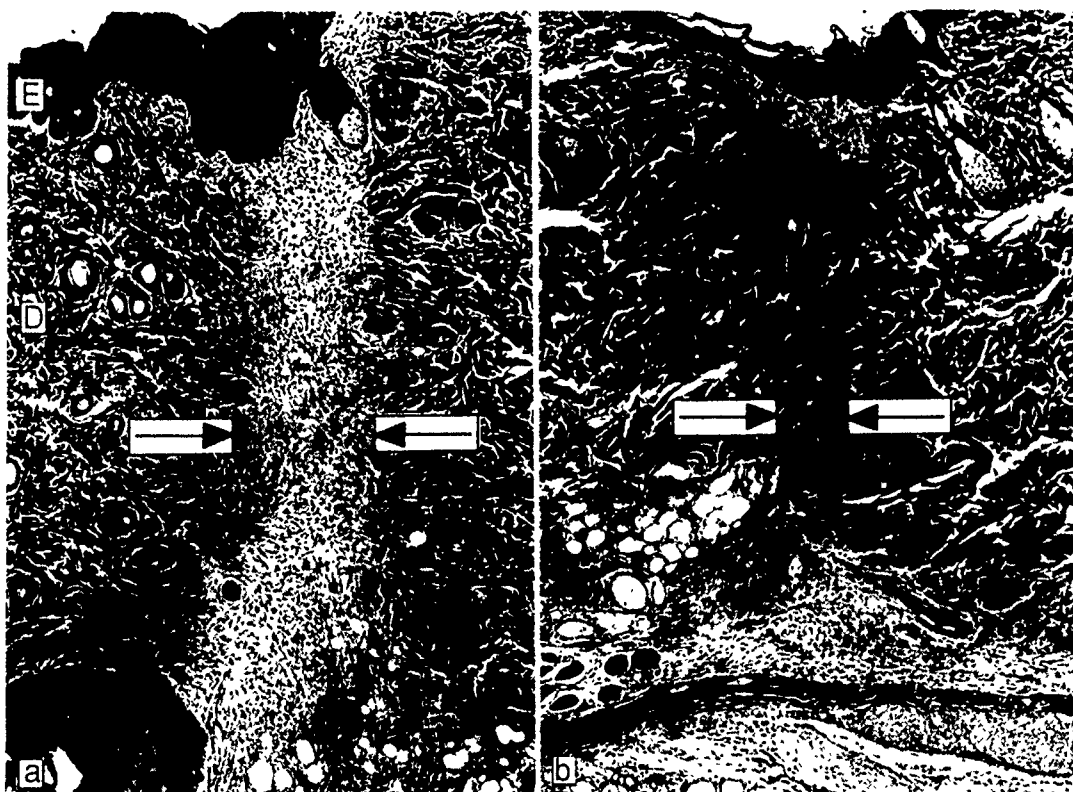
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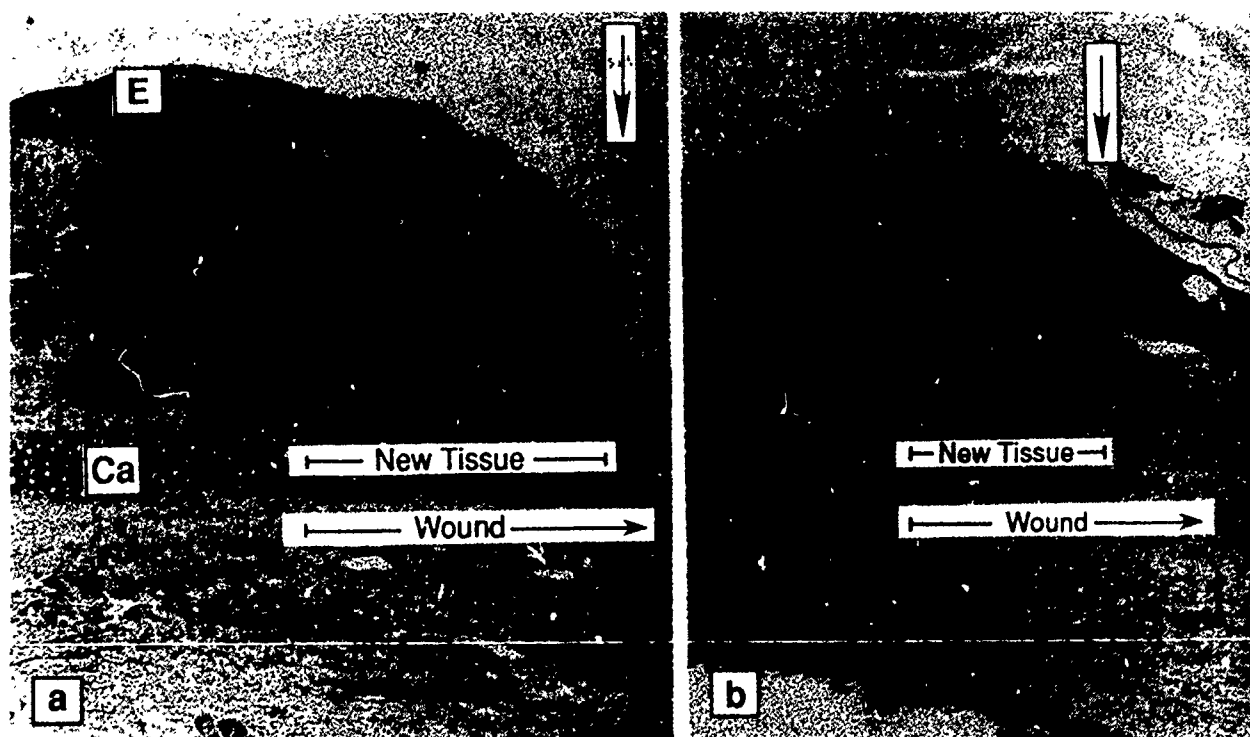
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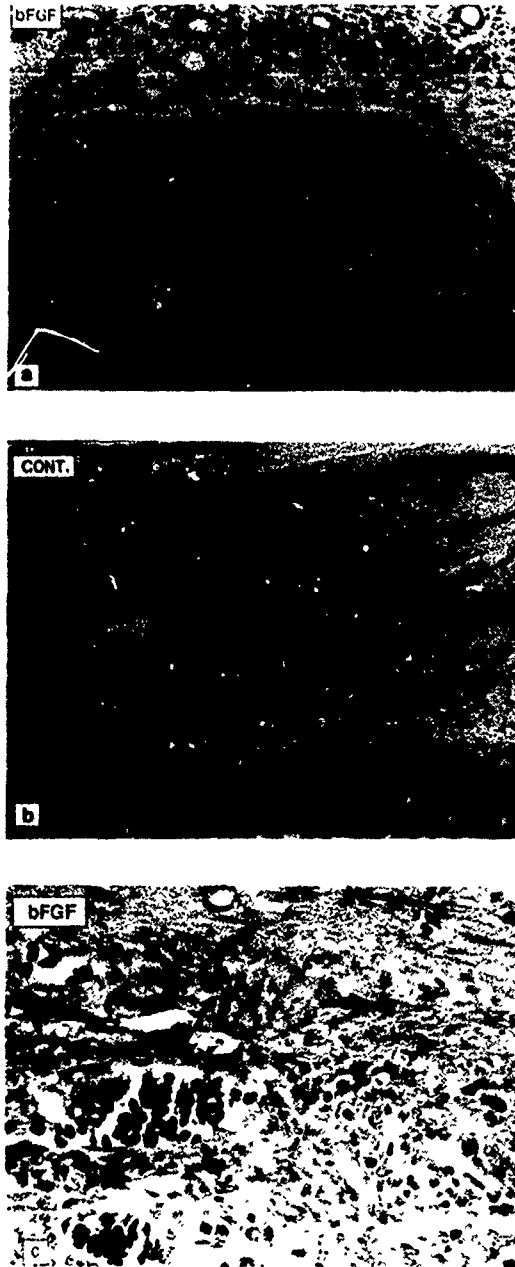
Figure 4

CURRENT STATUS OF BONE MORPHOGENETIC PROTEINS AND OSTEOGENIN

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ABSTRACT

Implantation of demineralized bone matrix in extraskeletal sites results in local bone morphogenesis. Bone morphogenesis is a sequential multistep process that includes chemotaxis, mitosis, and differentiation. Recently a family of bone morphogenetic proteins (BMP's) have been cloned by recombinant DNA techniques ane expressed. The members include osteogenin (BMP-3) and osteogenic protein 1 (BMP-7). The others are BMP-2, BMP-4, BMP-5, and BMP-6. The availability of recombinant proteins has set the stage for investigation of the mechanism of action of BMP's and their potential clinical applications. The future prospects are bright for fabrication of prostheses with bone morphogenetic proteins to ensure optimal implant-interface cellular and molecular interactions.

INTRODUCTION

Bone has considerable potential for repair and regeneration. The potential of allogeneic demineralized bone matrix to induce new bone formation in rats is well known.[1-1] Bone induction is a sequential multistep developmental cascade and consists of the following steps: chemotaxis of progenitor cells (days 1 - 2), mitosis of mesenchymal cells (day 3), differentiation of chondroblasts and chondrocytes (days 5 - 7), hypertrophy and calcification of cartilage matrix (day 9) with angiogenesis and vascular invasion (day 10), bone formation (days 10 - 11), bone remodeling (days 12 - 18) and hematopoiesis in the newly formed ossicle (days 20 - 21). The demineralized bone matrix-induced osteogenesis is reminiscent of embryonic limb morphogenesis in the fetus and stages of fracture repair in adults.

ISOLATION AND CLONING OF OSTEOGENIN AND BONE MORPHOGENETIC PROTEINS

The demineralized bone matrix is the solid state. In order to isolate bone inductive proteins the isoluble matrix must be solubilized. The matrix was dissociatively extracted by chaotropic reagents such as 4 M guanidine hydrochloride, 8 M Urea and 1% (w/v) sodium dodecyl sulfate.[4] The soluble fractions when reconstituted with insoluble collagenous matrix and implanted in rats induced new bone formation.[4] The bone inductive proteins from humans, monkey, bovine, and rat are homologous and appear to be equipotent in rats.[5] The bone inductive protein, osteogenin was isolated by heparin affinity chromatography and preparative gel electrophoresis.[6] The bone inductive activity was localized to the zone between 30-40 kilo Daltons (kDa). Upon reduction in dithiothreitol and mercaptoethanol a broad band of 22 kDa was observed; however, the biological activity was lost indicating that the dimeric molecule is bioactive. The amino acid sequence of tryptic peptides of osteogenin was similar to bone morphogenetic protein 3 (BMP-3). Bone morphogenetic proteins BMP-2A, BMP-2B and BMP-3 have been cloned and expressed by Wozney and colleagues.[7] Recently, a novel gene, osteogenic protein 1 has been cloned.[8] Table 1 summarizes the sequence homology of the carboxy-terminal quarter domain of the osteogenin-bone morphogenetic protein family. These are related to the transforming growth factor β (TGF-β) superfamily. It is noteworthy that bone morphogenetic proteins are related to developmentally important regulatory genes such as decapentaplegic (dpp) in Drosophia in Vgl in amphibians such as Xenopus laevis.[9,10] Recent explosive advances in mesoderm induction in Xenopus have implicated activins.[11] Activins and inhibins were earlier postulated to play a role in follicle stimulating hormone (FSH) release by anterior pituitary gland. It is indeed exciting to contemplate that seemingly disparate biological functions as bone morphogenesis in man, mesoderm induction in amphibians and pattern formation in fruit flies are regulated by members of the transforming growth factor β superfamily. The growing list of novel BMP's has expanded to include BMP-5 and BMP-6.[12]

STIMULATION OF OSTEOBLASTS AND CHONDROCYTES

Purified homogeneous osteogenin stimulated alkaline phosphatase activity and collagen synthesis in periosteal cells and osteoblasts derived from fetal calvaria in rats. The production of sulfated proteoglycans was stimulated in rat fetal chondroblasts and rabbit articular chondrocytes. Osteogenin increased the number of alkaline phosphatase positive colonies.[13]

RECEPTORS FOR OSTEOGENIN AND BMP-2B

As a first step towards defining responsive cells and receptors for bone morphogenetic proteins we investigated the binding and localization of radioiodinated osteogenin by autoradiography in developing rats.[14] Maximal binding was demonstrated in mesodermal tissues such as perichondrium, cartilage, periosteum and bone. These results are suggestive of a developmental role for osteogenin in skeletal morphogenesis.[14] Very recently similar results were obtained with recombinant bone morphogenetic protein 2B (S. Vukicevic and V. Paralkar unpublished). The molecular mechanism of action of BMP's is not known. As a first foray we have examined BMP-2B responsive cells for the presence of specific cellular binding proteins. Specific high-affinity binding proteins (receptors) were identified for BMP-2B in MC 3T3 E1 osteoblast-like cells.[15] Transforming growth factor β did not compete for the binding of radiolabeled BMP-2B. Chemical crosslinking of radiolabeled BMP-2B demonstrated two components at 200 and 70kDa in osteoblast-like Mc 3T3 E1 cells. Scatchard analysis of the binding data showed a high-affinity receptor with an apparent dissociation constant of 128 ± 40 pM.[15]

OSTEOGENIN INTERACTS WITH TYPE IV COLLAGEN

During the course of experiments designed to assess the binding of radioiodinated osteogenin to various extracellular matrix macromolecules we revealed the unexpected finding of high affinity for type IV collagen.[16] The binding occurred at physiological ionic strength and was reversible. It has recently been demonstrated that transforming growth factor β1 also binds to type IV collagen avidly.[17] It is likely that during vascular invasion prior to osteogenesis osteogenin and related BMP's bind to type IV collagen and are oriented to an optimal biological conformation to initiate bone formation concentrically around blood vessels.

OSTEOGENIN REGENERATES CALVARIAL NON UNION

The availability of osteogenin and recombinant bone morphogenetic proteins will permit the rational design of biomaterials for othopaedic surgery, plastic and reconstructive surgery and dental surgery. The efficacy of purified osteogenin to heal completely critical size defects in rat calvarium was demonstrated.[18] The stage has been set for systematic preclinical and clinical investigations.

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<u>OSTEOGENIN / BMP - TGF-ß SUPERFAMILY</u>

PRE	PRO	ACTIVE

	$\%$
Osteogenin (BMP 3)	100
BMP 2A (BMP 2)	49
BMP 2B (BMP 4)	48
BMP 5	37
BMP 6	43
OP 1 / BMP 7	37
TGF-ß 1,2,3,4,5	~34
Vg 1	49
Activin $ß_A$, $ß_B$	37
Inhibin	30
DPP	43
MIS	42

CLINICAL APPLICATIONS
OF MOLECULAR ENGINEERING:
BONE AND CARTILAGE REPAIR

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ABSTRACT

Over the past decade, the field of molecular biology has given rise to the development of the applied discipline of molecular medicine. Based on recent applications of recombinant DNA technology, genetic mapping analysis and other investigational tools to connective tissue research, the physician and surgeon is now ready to begin using molecular engineering in the diagnosis and treatment of musculoskeletal disease. This article reviews the history of scientific exploration in molecular biology as it applies to bone and cartilage repair. Clinical cases are presented to show how the fruits of scientific knowledge may be brought to bear on some of the more challenging musculoskeletal problems. Although some of these examples may prove to be accurate representations of how molecular engineering will be used in specific clinical settings, the purpose of this review is to provide an orientation and philosophical approach to the applications of molecular engineering in traumatic and reconstructive surgery of the skeleton.

INTRODUCTION

The growth of scientific information appears to develop in a logarithmic-like fashion as opposed to a linear one. Each new piece of data acquired and each new technique developed leads to advances in scientific knowledge which accelerate technology by orders of magnitude. This growth of knowledge in the areas of bone and cartilage repair will soon be realized in our clinics and operating rooms. As the close of the twentieth century gives way to the next millennium, we stand on the precipice of the new age of musculoskeletal medical science.

To begin to understand how new scientific advances might be clinically applied, it is important to identify the incidences and associated costs of the most commonly occurring conditions which require medical and surgical intervention. According to recent studies, it is estimated that approximately 200,000 bone graft operations[1] and approximately 265,000 total joint procedures[2] are performed in the United States each year. In addition, 1.5 billion fractures are sustained annually in osteoporotic patients.[3] The cost to the American economy of osteoporosis alone is in excess of ten billion dollars.[3]

Since cost has become a major factor in the allocation of medical services, scientists, universities, and biotechnology companies are now finding that cost plays an important role in directing research and development. However, it is important to understand that cost is measured in many ways. Work loss, early retirement, the effects of emotional stress and finally, monetary loss, are among the costs incurred by a medical condition. It is therefore necessary to consider all costs when allocating expenditures to the study and treatment of specific diseases. It can be anticipated that the results of ongoing federally-driven outcome studies will determine which technologies are most cost-effective[4]. These findings will almost certainly lead to decisions on how research dollars are allocated in the future.[4]

HISTORICAL PERSPECTIVES

The progress of research on bone and cartilage repair has been punctuated by the important contributions of certain key investigators (Table I). One of the first pioneers in this area was a late 19th century surgeon named Senn. Using decalcified bone from the ox tibia as a delivery system for an antiseptic (iodoform), Senn attempted to treat osteomyelitis in the dog skull.[5] Although his purpose was to eradicate the infection, he reported the healing of these bone cavities through a poorly understood mechanism of induced "osteogenesis."[5] Later, in 1931, Huggins described the formation of bone in a subcutaneous site under the influence of implanted epithelium from the urinary tract.[6] This and subsequent studies resulted in the first experimental models for induced osteogenesis.[6,7]

In 1965, Marshall R. Urist demonstrated the induction of ectopic new bone in laboratory animals by the implantation of demineralized bone matrix.[8] This report

paved the way for a new era of musculoskeletal research during which numerous investigators explored further the phenomenon of "bone induction" or "induced osteogenesis." By the early 1980's, clinical investigators had begun using demineralized human allogeneic bone in the treatment of osseous defects in patients. Clinical use of this demineralized bone matrix was applied to reconstruction of maxillocraniofacial deformities,[9,10,11] phalangeal cavities in the hand[12] and in the augmentation of spinal fusions.[13] Today, this bone graft substitute material (DBM) is commercially available from several companies.

By the end of the 1980's, orthopaedic trauma surgeons at UCLA Medical Center, under the scientific direction of Marshall R. Urist, implanted partially purified human bone morphogenetic protein into phalangeal cavities,[14] nonunion defects in the tibia[15] and femur[16] and eventually fresh traumatic defects in the tibia.[17] Some of these operations were combined with local and free-flap soft tissue procedures to optimize the biological environment of the host bed. The early results demonstrated healing capacities similar to (and in some cases, better than) autogenous bone graft procedures with a much lower incidence of morbidity. This was due to the lack of a need for harvesting autogenous bone from other skeletal sites.

Because it was recognized that the yield of bone morphogenetic protein from cadaveric allogeneic bank bone was limited and because it was learned that this inductive protein may work in a more optimal fashion when it is implanted along with other specific growth factors, efforts were made to develop recombinant technologies for the production of sufficient quantities of highly specific factors. The scientific contribution of Wozney and co-workers[18] was an important step in that direction.

CLINICAL APPLICATIONS

Based on the early clinical results of induced osteogenesis, clinicians can now envision a variety of applications of bone morphogenetic proteins as well as other biological factors. To imagine how these factors may be applied, it is necessary to identify the clinical conditions which would benefit most from their use. In addition, these applications must be considered in relation to the alternatives which are presently available. Table II shows a partial list of those conditions for which the application of biological factors may become important. Since an approach to improving upon the

treatments of these conditions must be guided by specific goals, it is necessary to identify what it is that the clinician requires in order to achieve these objectives.

The factors whose applications are reviewed in this article are specific polypeptide products of cells that can function as bone and/or cartilage growth factors, hematopoietic colony stimulating factors, attachment-promoting proteins, or other biological response modifiers which induce specific target cells or affect the response of these cells to other stimuli. However, in any given clinical setting, the responses which the clinician would like these factors to modulate are not necessarily of equal importance. Therefore, it is necessary to prioritize these responses by first identifying clinical "need " and following this by the identification of specific clinical "wants."

In terms of "needs," the clinician and/or scientist must begin with the premise that a wound will not heal and a bone will not grow unless there exists an optimal systemic environment, an optimal systemic response, an optimal local environment, and an optimal local response. It would therefore be desirable to have both locally and systemically injectable[19] or implantable[20] materials which could ensure that these conditions will be present (eg. normalizing the systemic and local response to a soft tissue wound in the foot of a patient with brittle diabetes mellitus). If technology could accomplish this goal and provide for these needs, it might become apparent that the human organism already has the necessary biological mechanisms for healing most conditions.

Beyond the question of "needs" of course, is the question of "wants." Here the clinician is dealing with a situation whereby the biologic response desired is beyond the capacity of the human system even under the most optimal set of conditions (eg. regenerating a dead femoral head or replacing a large hyaline cartilage defect in a joint). Factors which one would "want" to have in order to accomplish these goals could include chemotactic, attachment, competence, progression, and inductive factors, as well as conductive surfaces, delivery systems and possibly pluripotent stem cells.

Chemotactic factors could be extremely valuable for the purpose of attracting the migration or ingrowth of progenitor cells to the area of a wound. Attachment factors could function not only to maintain these newly attracted cells in this wound repair environment but also to influence their proliferative capacity and optimize their phenotypic expression. Competence factors function to prepare a cell to undergo a

change in its cell cycle. Since most cells, with the exception of a few constantly dividing cell types, are in a resting (G_0) phase of their cycle, in order for a cell to divide, it must acquire the ability to respond to a specific signal. Competence factors function to change a cell's responsiveness to other signals thus allowing it to enter into a G_1 phase of the cycle during which time it is capable of division. Progression factors stimulate cell division and proliferation and result in the production of sufficient numbers of cells of a specific type to ultimately produce new tissue. Inductive factors stimulate differentiation and differentiated function and are thus of major importance in mediating wound healing and matrix production.

To use these factors in patients, it will be necessary to have a delivery system which will bring the factors into contact with the target cells, protect them from immediate degradation in the host, and control the kinetics of their release such that their timing of appearance, local concentration,[3] and inhibitory and stimulatory functions are properly coordinated. Finally, all of these factors could potentially lead to more rapid and predictable healing responses if the appropriate conductive surface were to be present and if immunologically modified, pluripotent stem cells were available for local implantation.

With these considerations in mind, the following clinical cases are presented to show how molecular engineering may be applied to musculoskeletal disease:

Case #1 - Tibial Non-Union

A forty-one year old healthy white female slipped on an icy sidewalk and sustained a comminuted fracture of her right distal tibia and fibula (Figure 1). Standard conservative orthopaedic management in a cast led to delayed union after nine months. Pulsed electromagnetic stimulation for an additional three months failed to result in healing. The fracture was ultimately treated with open reduction and internal fixation. Healing occurred four months later. However, as a result of the long term (app. 16 month) period during which time she was unable to put weight on her leg, significant disuse osteoporosis developed throughout the tibia (Figure 2). The patient required an additional eighteen months of intensive physical therapy in order to regain reasonably normal motion, bone mass and ambulatory function.

Comment

Prevention of non-union is perhaps the most obvious indication for the use of an osteogenic substance. If such a substance were to have been introduced into the original fracture in the above case, perhaps it would have healed within the expected time frame of three to four months. The patient would have been back to her normal activities within four or five month's time.

Currently, the Food and Drug Administration appears to be focusing on patients with existing non-unions as prime subjects for inclusion in studies to test the effects of osteogenic substances. While it is understandable why these patients are reasonable subjects for a clinical trial of this nature, it must be recognized that the response of a non-union to an implanted osteogenic substance may be entirely different from that of a fresh fracture. In the former, the host bed consists of predominantly inactive mature fibrocartilaginous tissue while in the latter, the host bed consists of a fresh hematoma in the midst of an acute inflammatory response. These differences could play significant and important roles in determining the types of cells present and the nature of their responses to injectable or implantable substances. Based on a knowledge of which types of fractures are at greatest risk for developing problems with healing, it should be possible to identify these fracturs at the time of injury and intervene in a prophylactic manner using the appropriate inductive therapy.

Case # 2 - Articular Cartilage Defect

A twenty-four year old healthy white female fell down a flight of steps and sustained a direct blunt contusion to her right knee. Eighteen months later, she continued to experience significant medial joint line tenderness. An arthroscopic examination showed a 1.5 cm diameter area of cartilage degeneration (chondromalacia) without any evidence of healing. Treatment consisted of arthroscopic debridement and drilling of the subchondral bone in a weak attempt to stimulate a reparative response which could potentially lead to fibrocartilage production. Post-operative management consisted of a continuous passive motion protocol and intensive physical therapy. Currently, the patient is mildly improved but still experiences pain in the area of the original injury.

Comment

Most studies have shown that hyaline cartilage has very limited potential for tissue repair and almost no ability to heal large defects. When articular cartilage injuries extend through the entire thickness of the cartilage and extend into subchondral bone, any healing response which is seen is usually initiated by the subchondral osseous tissue. Some reports suggest that motion of an injured joint in a continuous passive manner results in the formation of fibrocartilage which can provide articular cartilage function.[21] How well tissue forms in large defects and how well it functions in the long-term remains an unanswered question.

With the advent of advanced arthroscopic surgical techniques and the development of new instrumentation, orthopaedic surgeons are now capable of doing many operations in joints using relatively atraumatic approaches. If a substance were available which could stimulate chondrogenesis, it should be possible to introduce it into the injury site in a joint with minimal surgical trauma. In a case such as the one presented above, one could envision a scenario whereby the patient undergoes an arthroscopic procedure in which the host bed is prepared by surgical debridement, followed by the coating of the exposed bone surface with an attachment-promoting material (e.g. laminin-containing, RGD-containing), and the subsequent application of cultured chondrocytes embedded in a chondrogenic delivery system. This would be followed by rehabilitation protocols using continuous passive motion and other physical modalities to enhance the healing and functional recovery.

Case # 3 - Total Hip Arthroplasty

A thirty year old black female with sickle cell anemia presents with hip pain of one year's duration and an X-ray showing Stage IV osteonecrosis of the left femoral head (Figure 3). All attempts at conservative management have failed and no surgical procedure which would leave her own femoral head intact would appear to be effective. A total hip replacement is performed with a noncemented, porous-coated prosthesis. The post operative X-ray (Figure 4) shows distal intimate contact between the stem and the endosteal surface of the femoral cortex. Proximally, the degree of bone-implant contact is difficult to assess.

Comment

This case describes an all-too-commonly occurring situation in which the only reasonable therapeutic option for a young patient with a diseased hip joint is an artificial joint replacement. While total hip replacement arthroplasty has been an extremely successful operation in the treatment of elderly patients with osteoarthrosis, its use in young patients has been much less successful. This has been attributed mainly to the fact that the long lasting durability of cemented implants is usually limited to not more than ten to fifteen years. While the new age of porous-coated hip implant technology has offered the hope that these implants will perform in a superior manner over time, the long term results are not yet available. It is presently thought that two factors which may influence these results will be, 1) the ability of bone to grow into the surface of the prosthesis, and 2) the way this ingrowth affects the stress environment and remodeling response of the skeleton. Biologically, one can only expect bone to grow into a prosthesis when the contact between the two is sufficiently intimate.

As examples of how a lack of bone ingrowth could potentially lead to problems, consider the radiographs of the two cases shown in Figures 4 and 5. Figure 4 shows grossly evident contact between the distal one-third of the stem and the intramedullary canal of the femur. However, proximally, contact is questionable. The long-term results of bone remodeling could potentially lead to bone hypertrophy distally with disuse osteoporosis proximally. This could result in a biomechanical cantilever system whereby proximal motion and distal fixation leads to fatigue fracture of the metal implant. In Figure 5, another young patient underwent a similar operation in which very little contact between the prosthesis and the bone existed anywhere. This implant remained unstable and ultimately required revision surgery.

The ability to obtain intimate contact between a prosthesis and the bone, and to do it in the appropriate places, is beyond the capability of even the most skilled surgeon. The development of a material which could be applied to a prosthesis to promote bone ingrowth, even without close and intimate contact, would make the technology of noncemented joint replacement surgery potentially much safer and reliable. Since bone remodels around such implants in response to mechanical forces, the ability of the surgeon to determine where bone ingrowth should occur and where it should not, may enhance his or her ability to control the stress environment and optimize the results.

Presently available materials such as hydroxyapatite coatings and different types of surfaces are only passively conductive and probably inadequate for achieving these goals.

Case #4 - Traumatic Osteonecrosis of the Hip

A thirty year old healthy white male suffered a traumatic dislocation of his right hip while playing softball. An attempted closed reduction in a local emergency room converted this injury to a fracture dislocation (Figure 6). Open reduction and internal fixation was performed however, three months later, there was no evidence of healing and a bone scan showed photopenia of the right hip consistent with ischemic necrosis (Figure 7). As a result, this young patient underwent hip arthroplasty with a bipolar prosthesis.

Comment

While the treatment of traumatic ischemic necrosis of the femoral head is still controversial, most femoral head-sparing procedures require that some significant portion of the femoral head remain alive. In this case, it is assumed that the entire femoral head was necrotic and thus not salvageable in its present form. Some have advocated using vascularized fibular autograft transplants or bone-muscle pedicle flaps from the lesser or greater trochanters but the successes of these procedures are unclear at this time. A more predictable and successful surgical outcome could potentially result if these vascularized transplants could be combined with growth factors and perhaps pluripotent stem cells. One could envision a procedure in which the necrotic femoral head is excavated such that it is hollow with the exception of a thin shell of subchondral bone and articular cartilage. Into this shell could be implanted a vascularized fibular autograft or local bone-pedicle flap. This graft could then be surrounded by a composite of osteoprogenitor stem cells, inductive proteins, and perhaps a delivery system which involves extracellular matrix proteins and conductive substrates to enhance the repair process.

BIOLOGIC RESPONSE MODIFIERS: EARLY CLINICAL RESULTS

A variety of immunomodulatory proteins and hematopoietic colony stimulating factors have been used in the care of cancer and immunosuppressed patients. Several

of these factors have undergone testing in phase I and phase II clinical trials and others have even achieved FDA approval for specific indications. Among these are the interferons (α, β, and γ), which have been used to treat tumors, viral infections, juvenile or laryngeal papilloma, and autoimmune disease; and Interleukin-2 which has been used to treat melanoma and renal cell carcinoma in combination with lymphocyte activated killer cells. Certain colony stimulating factors such as granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor have achieved FDA approval for the treatment of certain immunosuppressed patients, patients with failed bone marrow grafts and chemotherapy-induced leukopenia. Interleukin-1 and tumor necrosis factor are still in phase I trials.

The preliminary clinical results from studies using these biologic response modifiers in the types of patients described above, will pave the way for their potential applications in the treatment of musculoskeletal disease. Not only has it been shown that many of these factors are involved in bone and cartilage formation, turnover, and degradation, but specific metabolic bone diseases may be related to deficiencies of one or more of these factors. Recent evidence that a defect in the structural gene responsible for the production of macrophage-colony stimulating factor (M-CSF) is present in animals with a specific form of osteopetrosis,[22] combined with evidence that treatment of these animals with M-CSF cures this condition,[23] is an example of how a metabolic bone condition may respond to a hematopoietic colony stimulating factor. Further evidence that targeted disruption of the c-src proto-oncogene by homologous recombination leads to osteopetrosis in mice[24] may provide evidence to support the exploration of new genetic pathways of investigation of metabolic bone disorders.

QUESTIONS AND CONCERNS

An important part of the development of any new medical technology is the careful and systematic evaluation of potential toxic effects. While some of these effects may be manifest as acute short-term problems, a much greater concern exists over the possibility of patients developing serious complications which may become obvious only after months or years of treatment. With regard to the clinical experiences using some of the biologic response modifiers, examples of these effects include fever, hypotension, hepatic and renal failure, myocardial infarction, capillary leak syndrome, and massive edema, to name a few.

Specificity is another important consideration in the development of a new therapeutic agent. The greater the specificity of a factor for its target cell, the greater control the clinician has over the patients' response. Furthermore, a specific biologic response, such as a wound healing process, may require the participation of several types of cells at different points in time. Therefore, the specificity of a factor may change at different times during the response period. The need to develop the appropriate delivery systems which not only protect factors from degradation but also optimize their concentrations and kinetics of release may be critical to the success of any molecular product. Finding the optimal combinations of factors may also be critical to the success of this technology.

Although not yet observed, an ever present concern with regard to growth factors, is their potential cancer producing effects. It would seem intuitive that any factor which has the ability to change a cell's behavior could possibly lose control over that cell and this could lead to the induction of a neoplastic process. One of the most important concerns with regard to the use of molecular engineering in treating diseases and developing new drugs, will be the ability to interrupt an induced process once it has been initiated.

EXPECTATIONS FOR THE FUTURE

How well the human organism will respond to cells, growth factors and other biological materials remains to be examined. While the responses in lower mammals have been encouraging, and while significant clinical effects have been observed in patients who have been treated with recombinant biologic response modifiers, the application of recombinant osteogenic or chondrogenic factors in patients has not been tested extensively.

Teleologically, it is recognized that bone regeneration in humans is possible. This is exemplified by the phenomenon of normal fracture healing by endochondral ossification. In this process, new mature bone actually regenerates in response to an injury. An extension of this phenomena which has been exploited in patients is the bone which is regenerated during distraction osteogenesis using the methods originally developed by Ilizarov.[25] In these cases, significant gaps and segments of the skeleton have been restored by surgical and mechanical manipulations.

One of the most impressive, and for the purposes of this discussion, interesting pathological conditions to affect musculoskeletal system is fibrodysplasia (myositis) ossificans progressiva (FOP). This is a rare autosomal dominant disorder characterized by symmetrical congenital malformations of the blastemal anlage of the hands and feet and by the progressive heterotopic chondrogenesis and ossification of soft connective tissues.[26] It is unknown what triggers this mechanism (although it has been suggested to be related to an injury and repair process) and no treatment is known. It is an extremely disabling disorder particularly because the ossified soft tissues generally take the form of fascial planes associated with specific muscle groups. In many cases, this leads to the partial or complete paralysis of the involved joints. Figure 8 shows an ossified iliopsoas muscle crossing the groin of a twenty-four year old woman with FOP. This patient is completely unable to flex or extend her hip joint.

A recent review provided strong evidence to suggest that FOP is a genetic disorder characterized by a disturbed developmental expression of the endochondral program and represents a mutation resulting in a dominant gain of function.[26] It was shown that an array of developmental gradients (characteristic patterns of disease expression) similar to developmental anomalies induced by pleiotropic mutations of the decapentaplegic (dpp) locus in Drosophila melanogaster may be related to a 75% sequence homology between the protein encoded by the dpp locus in Drosophila and the C-terminal region of two recently cloned human bone morphogenetic proteins (BMP-2A, BMP-2B), members of the TGFβ superfamily. It was therefore suggested that the genetic predisposition to develop this phenotype in humans may be related to bone morphogenetic protein expression.[26] This case provides evidence that it may be possible to obtain, in humans, a significant and even massive osteogenic response to a bone morphogenetic protein-like factor. It also suggests that an array of responses in humans may be possible by the proper molecular engineering of other factors in the TGFβ superfamily or by other related peptides.

Much has been learned over the past ten years concerning the technologies discussed in this review. The need to develop these advances into safe and medically effective therapeutic regimens is the challenge to today's investigator. The ability to provide these technologies to all patients rich and poor will require the cooperation of scientists, physicians, economists and government officials. As we enter the age of molecular medicine, the future looks bright for the treatment of musculoskeletal injury and disease.

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FIGURE LEGENDS

Figure 1

Anterior-posterior radiograph of the right lower leg taken immediately after ankle injury in a forty-one year old female. Note the presence of a mildly displaced, severely comminuted fracture of the distal tibia and fibula.

Figure 2

Anterior-posterior radiograph of the same lower leg shown in Figure 1 taken 16 months after the initial injury. Note the presence of plates and screws which were used to fix this fracture 12 months after injury. Although fracture healing is evident, there is now significant disuse osteoporosis.

Figure 3

Anterior-posterior radiograph of the pelvis of a thirty-year old black female. Note the presence of Stage IV osteonecrosis of the left femoral head.

Figure 4

Anterior-posterior radiograph of the hip taken immediately after implantation of a non-cemented, porous-coated total hip system in the patient whose X-rays are shown in Figure 3. Note excellent distal bone-implant contact of the femoral component. Proximally, femoral bone implant contact is questionable.

Figure 5

Anterior-posterior radiograph of the same type of non-cemented, porous-coated total hip system shown in Figure 4. Note that in this patient, there is a complete line of radiolucency surrounding the entire femoral component suggesting poor contact between the implant and the bone.

Figure 6

Anterior-posterior radiograph of the right hip in a thirty year old male who had just undergone attempted closed reduction for a hip dislocation. This X-ray now shows the presence of a complete fracture-dislocation of the femoral head and neck.

Figure 7

Technetium-99m MDP bone scan of the pelvis of the patient shown in Figure 6. Note the increased isotopic activity in the acetabulum and trochanteric areas of the right hip with complete photopenia of the right femoral head. This study was interpreted as showing complete necrosis of the right femoral head.

Figure 8

Lateral radiograph of the hip in a patient with fibro-dysplasia ossificans progressiva. Note the presence of an ossified iliopsoas muscle originating in the pelvis, crossing the groin, and inserting on the lesser trochanter of the hip.

TABLE I – MILESTONES IN INDUCED OSTEOGENESIS

1889	Senn[5]	First clinical use of decalcified bone
1931	Huggins[6]	Induced osteogenesis by urinary epithelium
1965	Urist[8]	Induced osteogenesis by demineralized bone matrix (DMB)
1972	Reddi and [7] Huggins	Induced osteogenesis as model of cellular differentiation
1981	Glowacki, Mulliken et al[9,10,11]	First successful use of DBM in patients
1988	Wozney et al[18]	Recombinant production of BMPs

TABLE II – CLINICAL APPLICATIONS
OF BIOLOGICAL FACTORS

<u>Condition</u>	<u>Present Alternatives</u>
Fracture Healing	Autograft/Allograft Ceramics Distraction Osteogenesis
Cartilage Repair	None (Continuous Passive Motion?)
Osteoporosis	Antiresorptive Drugs
Implant Stabilization	Autograft/Allograft Ceramics
Osteonecrosis	Core Decompression Vascularized Autograft Electrical Stimulation
Tumor Reconstruction	Allograft Metal Implant Distraction Osteogenesis Vascularized Autograft

TABLE II – CLINICAL APPLICATIONS
OF BIOLOGICAL FACTORS
(CONTINUED)

Joint Reconstruction	Allograft
	Metal Implant
Osteopetrosis	Bone Marrow Transplantation
Wound Healing in Debilitated Patients	Debridement/Skin Grafts
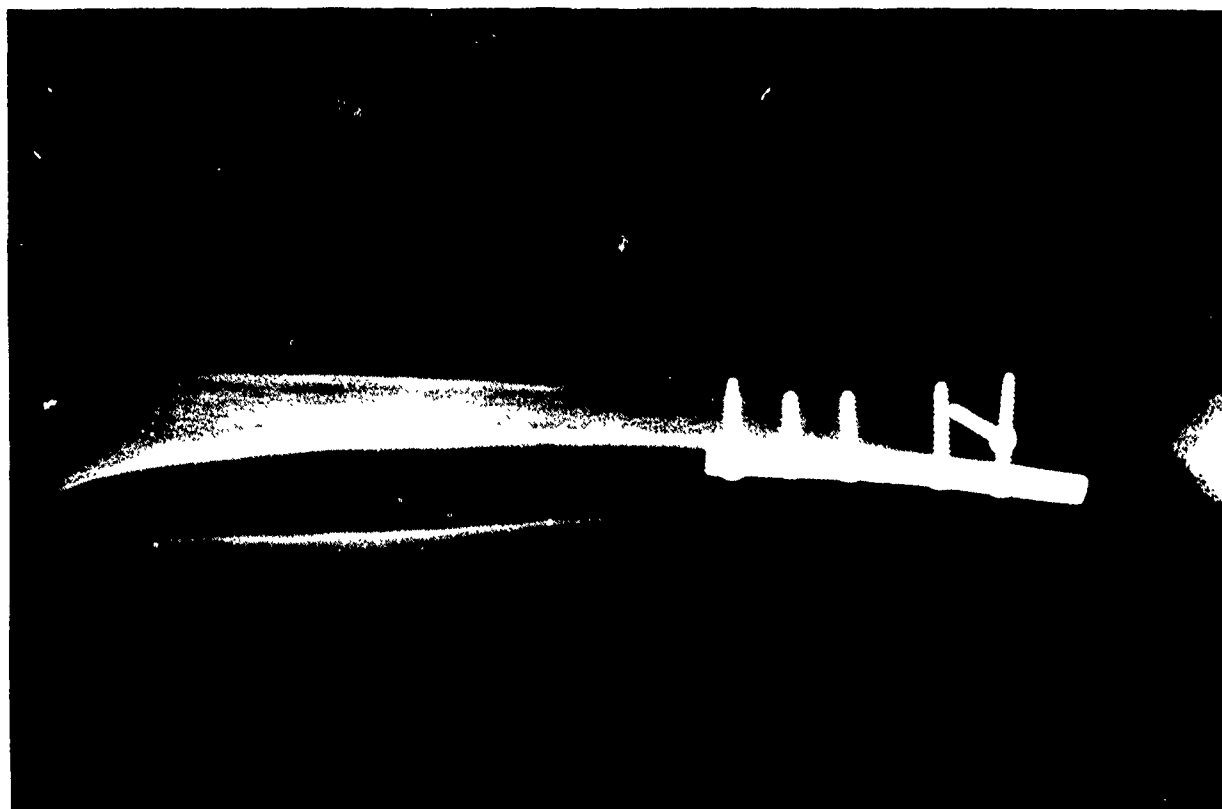
Figure 2

Figure 1
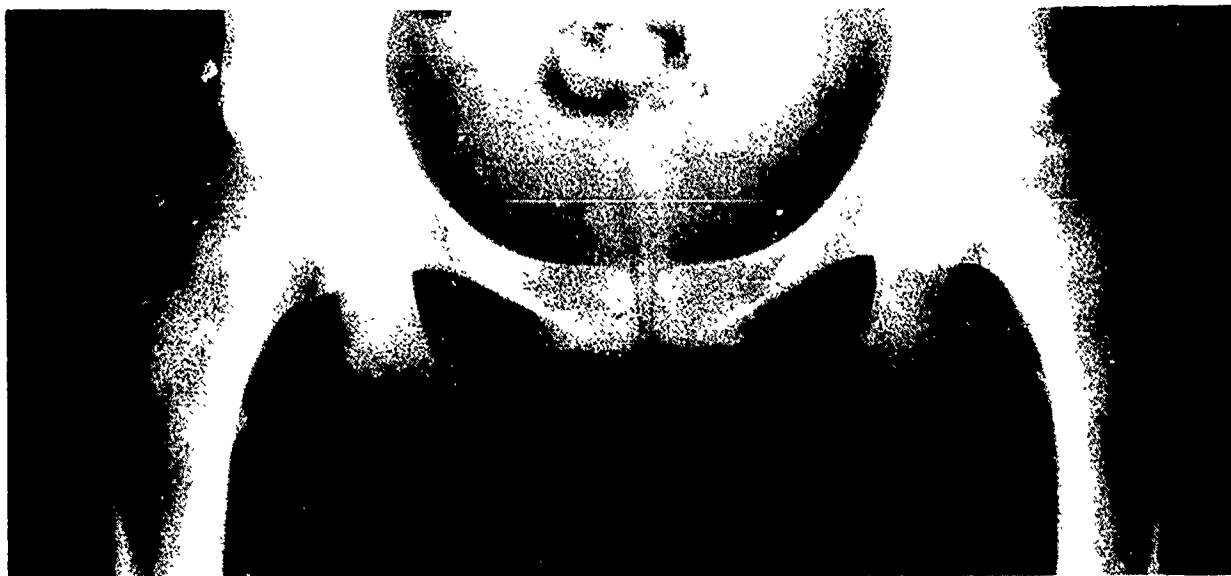
Figure 3
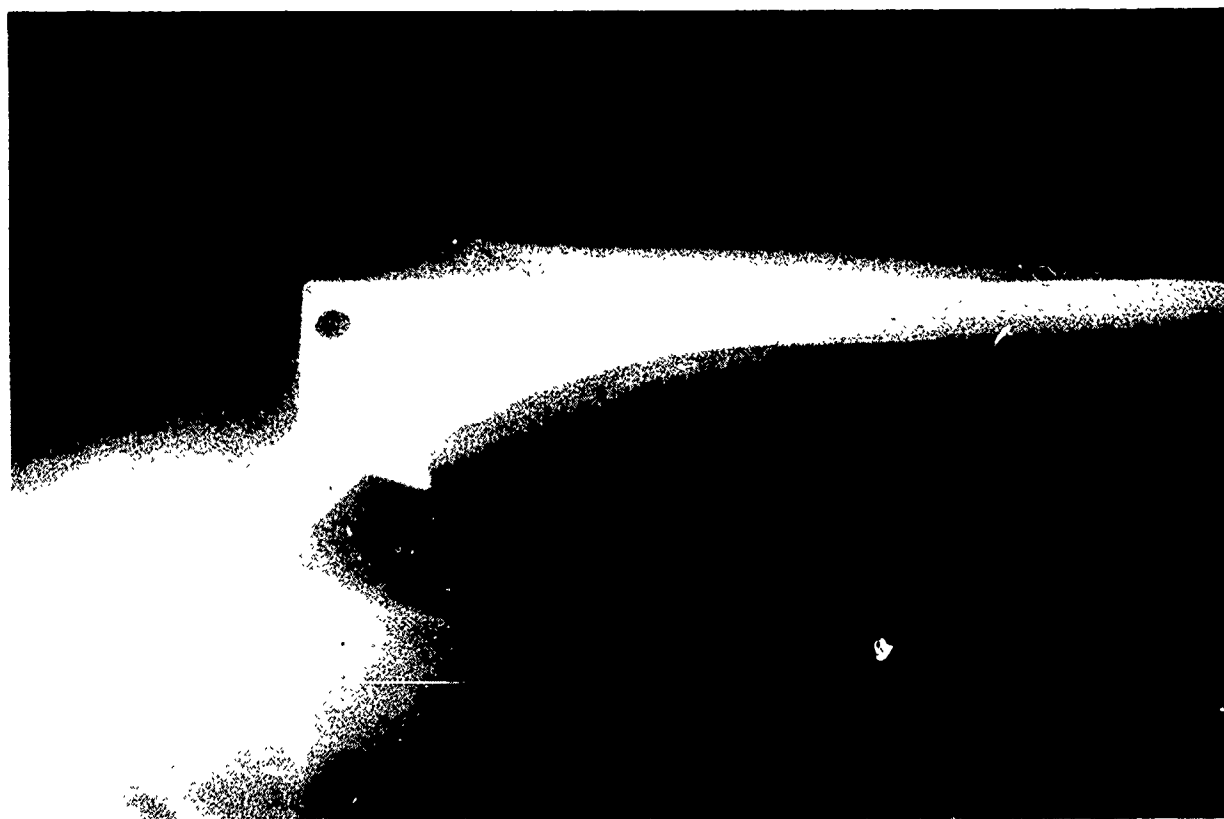
Figure 4

Figure 6

Figure 5
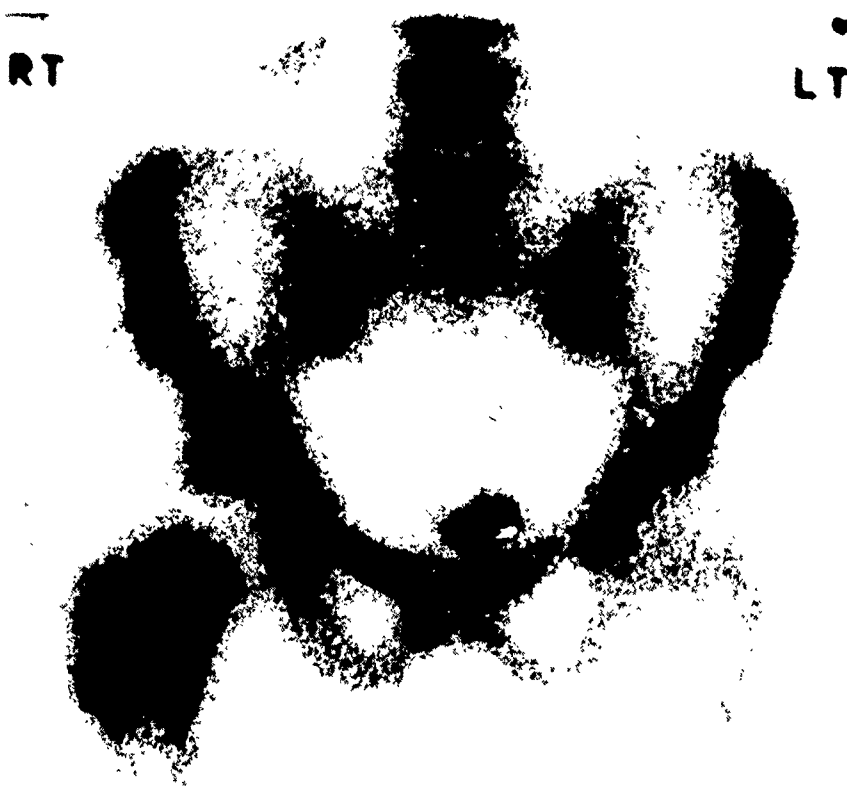
Figure 7

Figure 8

LABORATORY AND CLINICAL EXPERIENCE WITH A SYNTHETIC BONE GRAFT SUBSTITUTE

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ABSTRACT

The laboratory development of a synthetic osteoconductive matrix is described. The matrix when combined with autogenous bone marrow becomes an effective bone graft substitute. This material, named Collagraft™, has been utilized in a multicenter, randomized trial as an alternative to cancellous bone grafting in the treatment of acute fractures. The results of this trial are summarized. Future developments using this material are outlined and the initial laboratory data are introduced.

INTRODUCTION

With the exception of blood, bone is the most frequently transplanted tissue.[1] Bone grafts are needed in repair of fractures, arthrodesis and skeletal reconstruction following bone loss secondary to infection or neoplasm. In repair of fractures by internal or external fixation, bone grafting is needed when there is extensive comminution, segmental bone loss, and in high velocity or compound injuries.[2,3,4] In view of recent interest in aggressive management of fractures by internal and external fixation, it is not surprising that approximately 100,000 bone grafting procedures are performed each year in the United States.[5]

Currently, fresh autogenous bone is the most effective graft material available, most often using the iliac crests as the donor sites. Nevertheless, there are several disadvantages associated with use of autogenous bone, especially its limited supply at any one donor site and the added morbidity associated with its harvest.[6-9] As a result, numerous investigators have explored for potential as bone graft substitutes such diverse substances as homologous bone, demineralized allograft and synthetic organic

and inorganic constituents of bone. Synthetic materials are particularly attractive because they can be provided in infinite supply, they are easily sterilized and stored, and the significant risk of disease transmission by allograft tissues is avoided.[10]

The ideal graft substitute should supply the three elements of bone regeneration including an osteoconductive matrix, osteoinductive factors and osteogeniccells.[11]

Having all three properties, the synthetic substitute would be suitable for treating acute fractures, nonunion, and for reconstruction of large osseous defects. To date, many osteoconductive matrices such as coralline hydroxyapatite have been used in the acute fracture setting.[12,13] However, without combination with osteoinductors or osteoprogenitor cells, such matrices cannot be expected to be useful or predictable in the treatment of nonunions or large bone defects where the signals stimulating, inducing and modulating bone repair are turned off.

Over the last decade we have investigated the potential of a mixture of bovine collagen and a hydroxyapatite/tricalcium phosphate (TCP) fibrillar ceramic to serve as an osteoconductive matrix.[14-17] This matrix consists of soluble fibular collagen manufactured from bovine skin, currently marketed as Zyderm (Collagen Corp., USA). This provides a purely organic component which is rapidly remodeled.[14] To this is added a porous hydroxyapatite/tricalcium phosphate ceramic bead. The bead is approximately 65% hydroxyapatite and 35% tricalcium phosphate. The tricalcium phosphate phase is rapidly solubilized *in vivo* creating a porous granule with pores ranging in size from 0.5 to 1.0 mm in diameter. This matrix, when applied *in vivo* is, by itself, an effective osteoconductive lattice which encourages ingrowth of mesenchymal tissues. By itself it has no osteoinductive or osteogenic potential.[18,19]

In order to investigate the potential for this matrix to serve as a bone graft substitute, we have developed a rat femoral defect model for *in vivo* experimentation.[14,15,16,17] In this model the diaphysis of the animal femur is exposed subperiosteally. A polyethylene plate is affixed to the ventral surface with 1.3mm threaded pins. A section of femur is then excised, creating a defect measuring 5mm or approximately 20% of the bone length. If left ungrafted, this defect leads to nonunion in >90% of cases. The rigid plate maintains the coaxial relationship of the bone ends, allowing for introduction of test materials and permitting serial observation and quantitation of bone formation by radiographic measures (Figure 1). After sacrifice, the healed bone can be

easily mounted for biomechanical testing to analyze the mech. ¬al properties of the neoformed bone in the defect. We have used this model extensively to assay for bone formation when numerous osteoinductive, osteoconductive and osteogenic factors and materials are engrafted into the defect.

Werntz[14] extensively studied the usefulness of collagen as an osteoconductive matrix in the setting of this model. Collagen is the predominant protein found in the unmineralized matrix of bone. Collagen most likely serves as a structural framework for tissues undergoing mineralization, but its precise role in this process is uncertain. It is possible that the three-dimensional surface of collagen fibers provides nucleation sites for deposition of mineral.[20]

When placed in the femoral defect, collagen was found to be significantly inferior to cancellous bone as a graft material.[14] However, when mixed with autogenous marrow, collagen combined with marrow was found to be superior to cancellous bone. collagen alone apparently lacks osteogenic capacity but provides an osteoconductive matrix which, when combined with marrow, successfully leads to regeneration of bone in the defect.[21]

Calcium phosphate ceramics commercially prepared are stable with variable bioresorbability. They are generally composed of varying proportions of hydroxyapatite and tricalcium phosphate. The tricalcium phosphate is more rapidly resorbed, leaving a porous hydroxyapatite lattice. Studies by Klawitter and Hulbert[22] have indicated that the minimum pore size in porous ceramic which allows effective ingrowth is 100 microns. Most manufactured ceramics, including that produced by Zimmer Corporation and utilized in our studies, have pores ranging from 100 microns to 400 microns in diameter.

Ceramics also serve as osteoconductive matrices. TCP implants only form bone in the presence of marrow, but bone readily grows into ceramics placed adjacent to scarified cortex or into cancellous areas.[18] Ceramic beads, when added to solubilized collagen and marrow confer useful structural properties. The beads increase the viscosity, forming a paste-like consistency which impedes displacement of the material, thereby restricting the osteogenic and osteoinductive substance of the graft to the bone defect.[21]

A summary of the results achieved with various implant materials in the rat femoral defect model is shown in Figure 2. The combinations of collagen plus marrow

and collagen hydroxyapatite/tricalcium phosphate, plus marrow consistently yielded union rates of 90% or greater with biomechanical properties surpassing that obtained with a cancellous graft.

Based on these results, a bone graft substitute was developed for clinical trial.[23] The graft substitute was called Collagraft and consisted of a 1:1 mixture of fibrillar collagen highly purified from bovine dermis (95% type I collagen: 5% type III) and a porous calcium phosphate ceramic (65% hydroxyapatite and 35% tricalcium phosphate). To this mixture, 2.5cc of bone marrow aspirated from the iliac crest was added to each 7cc of Collagraft.

In this study Collagraft was utilized in a prospective, multicenter, randomized trial as a bone-graft substitute, comparing it to autogenous bone graft in the treatment of acute long-bone fractures treated by open reduction and internal fixation. The specific aim of this study was to test the hypothesis that Collagraft is comparable to autograft in achieving osseous union in acute fractures.

Eligible patients were between 18 and 70 years of age and had sustained a fracture of a long bone (humerus, ulna, radius, femur, tibia) within thirty days. Patients were excluded if they were severely obese, alcoholic, or had a history of infection at the fracture site. Patients receiving corticosteroids or other immunosuppressive agents and those with severe open fractures (IIIB and IIIC) were excluded. Patients were randomized by random block order to either group upon presentation. The rate and completeness of healing were assessed by an independent radiologist not involved in the study. To date, 139 patients have received Collagraft, while 128 have been grafted with autogenous bone. The groups are remarkably comparable in terms of age and sex distribution (Table 1). In addition the severity of injury and fracture locations are all comparable. After two years of follow-up, no difference in rate or completeness of healing can be detected between the two groups (Figure 3). Complications between the two groups are quite similar except for wound healing complications. The number of wound healing complications was higher in the autogenous bone group due to the 3.3% incidence of healing problems at the iliac crest donor site (Table 2).

These results led to the conclusion that Collagraft is a safe and effective bone graft substitute when applied to acute fractures. The major advantage of the material lies in the fact that the pain and morbidity of iliac crest harvest is avoided[9] and that Collagraft

carries no risk of hepatitis or HIV transmission. The material is also available in infinite supply. Collagraft does lack structural strength and, therefore, is not useful in situations where cortical grafting is needed. It may not be advisable to place it within joints, where it might migrate and interfere with joint function.

Current investigations are under way to evaluate ways to add osteoinductive potential to Collagraft. Bone marrow may have limited effectiveness as an osteogenic substance due to the small number of osteogenic cells found in a routine iliac crest aspiration.[24] Addition of a potent osteoinductive material might be more effective and make Collagraft suitable for expanded clinical applications such as nonunions and large bone defects. Furthermore, pharmaceuticals could be mixed with Collagraft creating a delivery system to treat bone disorders locally. Antibiotic-loaded Collagraft might be useful in the treatment of osteomyelitis, reducing the need for and risk of parenteral antibiotics. Collagraft loaded with antineoplastic agents could be utilized in the treatment of local bone neoplasm.

ONGOING STUDIES

The current investigative efforts in our laboratory are now focused in two areas. The first effort is work directed at testing osteoinductive growth factors which would be suitable to mix with Collagraft to expand its application and effectiveness in clinical settings. The second is directed at studies evaluating Collagraft as a carrier for the depot administration of antibiotic therapy for chronic osteomyelitis.

RhBMP-2 is a recombinant form of one of the osteoinductive proteins of the BMP family. Seven BMP molecules now produced by molecular cloning have been described.[25] All except rhBMP-1 appear to be members of the transforming growth factor-beta(TGF–β) supergene.[25-26] The osteoinductive proteins osteogenin and osteogenic proteins OP-1 and OP-2, are identical to BMP-3, rhBMP-7 and rhBMP-2.[27-30]

RhBMP-2 has been demonstrated to induce *de novo* cartilage and bone formation in the rat ectopic bone formation assay using subcutaneous implants.[26,31,32] In our laboratory we have applied rhBMP-2 to the rat femoral defect model using inactivated (i.e., guanidine extracted) rat demineralized bone as osteoconductive matrix. RhBMP-2 has been applied using a low (1.4mg) and high dose (11.0mg). Using our rat femoral defect model, we have shown that the high-dose rh-BMP2 implant yields significantly

higher union rate and earlier accumulation of calcified tissues in the femoral defect compared with deactivated bone matrix alone. Biomechanical testing of healed defects at 12 weeks demonstrated that the healed defects had comparable stiffness to intact femora only in the high-dose BMP group. Overall healing, bone formation, and development of mechanical strength were significantly better in the high-dose BMP–treated rats. Future studies will investigate addition of rhBMP-2 to clinical fractures both with and without an osteoconductive matrix.

Since the advent of total joint arthroplasty and widespread use of internal fixation devices, chronic ssteomyelitis has become a significant clinical problem.[33]Traditional treatment has called for radical debridement of infected bone and soft tissue with administration of parenteral antibiotics.[34-38] Treatment using parenteral antibiotic therapy is associated with numerous complications including nephrotoxicity, bone marrow suppression, ototoxicity and hypersensitivity. Furthermore, adequate blood levels of drug are a poor indicator of osseous concentration due to difficulty of penetration into necrotic bone.

In 1970, Bucholz[39-40] introduced the concept of localized depot administration of antibiotics using polymethyl methacylate for infected prostheses. Dalmers[41] proposed use of antibiotic-impregnated plaster of paris and other authors[42] have devised ingenious pump systems for depot/local administration of high levels of antibiotic therapy for treatment of chronic osteomyelitis.

In this study, a gentamicin-loaded hydroxyapatite bead was utilized as treatment for chronic osteomyelitis induced in New Zealand white rabbits infected according to the model of Norden.[43] A systematically treated control was utilized for comparison.

Chronic staph aureus osteomyelitis was induced in New Zealand white rabbits. A debridement and quantitative cultures were obtained at 21 days. Inoculated animals were then randomly treated with either systemically administered gentamicin or by implantation of gentamicin-loaded hydroxyapatite beads. After 28 days of treatment repeat cultures were obtained. All animals treated with gentamicin loaded hydroxyapatite beads were clinically non-infected, with quantitative cultures of bacterial load being <103 bacteria/gm of tissue, with no growth seen in 6 of 13 animals. All animals treated systematically remained clinically infected after 28 days of treatment, despite therapeutic MIC levels to the infecting organism (Table 3).

These data confirm the effectiveness of local administration of antibiotics using a hydroxyapatite ceramic as the carrier in the treatment of experimental osteomyelitis. Future studies will investigate the use of this matrix composite in the clinical setting in patients with chronic osteomyelitis and open fractures.

SUMMARY

In our laboratory we have demonstrated the effectiveness of a combination of collagen and a hydroxyapatite-tricalcium phosphate ceramic to serve as an osteoconductive matrix. In addition, we have early data which suggest that the addition of marrow and rhBMP-2 provides osteogenic and osteoinductive factors which improve the regenerative potential of the matrix. Collagraft has been shown to be an effective bone graft substitute in acute fractures treated by internal fixation. Future studies incorporating osteoinductors with Collagraft and antibiotic-loaded Collagraft for treatment of nonunion and infections are planned.

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FIGURE LEGEND

Figure 1

The in vivo rat femoral defect model. The defect of 5mm consistently leads to nonunion. Various graft materials placed into the defect can be assayed for bone formation, rate of union and biomechanical strength.

Figure 2

A summary of results of various combinations of graft materials utilized in the rat femoral defect model.

Figure 3

The independent radiologist's assessment of healing of the Collagraft and cancellous bone grafted patients.
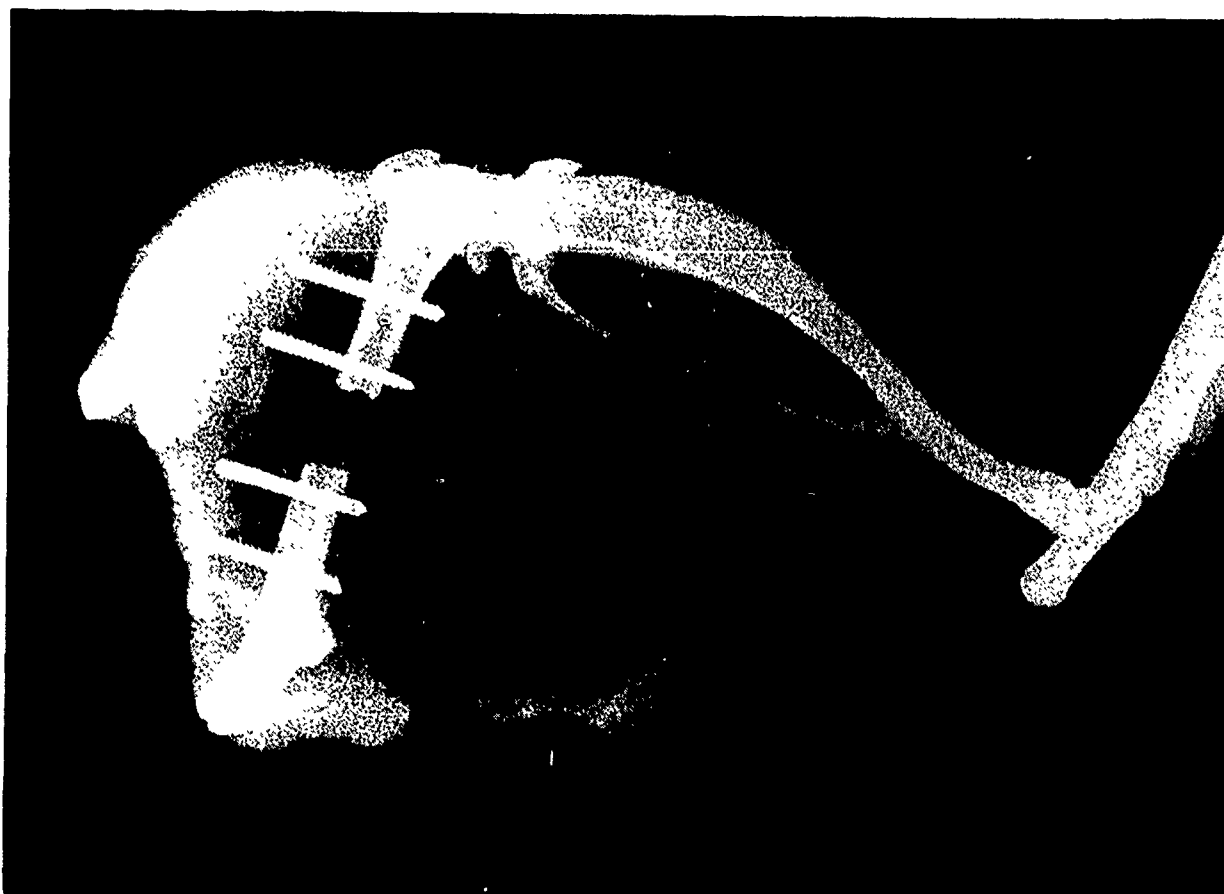
Figure 1

UNION RATE
(SCORE > 1)
THE EFFECT OF BONE MARROW
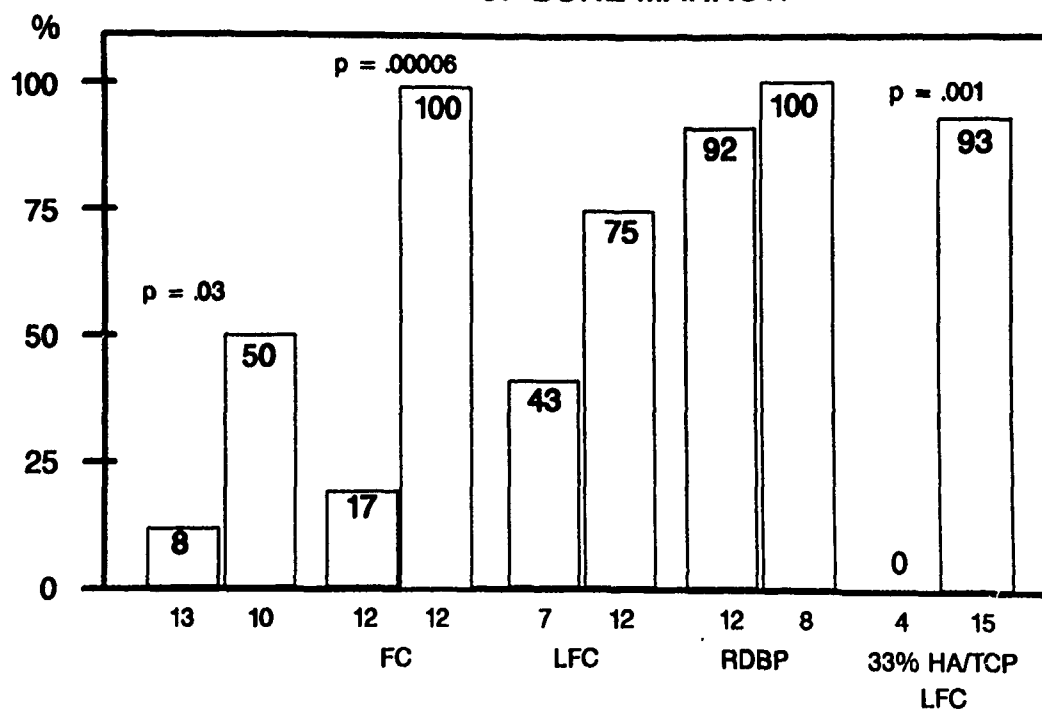
Figure 2

RESULTS OF INDEPENDENT RADIOLOGIST'S ASSESSMENT OF RADIOGRAPHIC HEALING

% HEALED

■ AUTOGRAFT   □ COLLAGRAFT
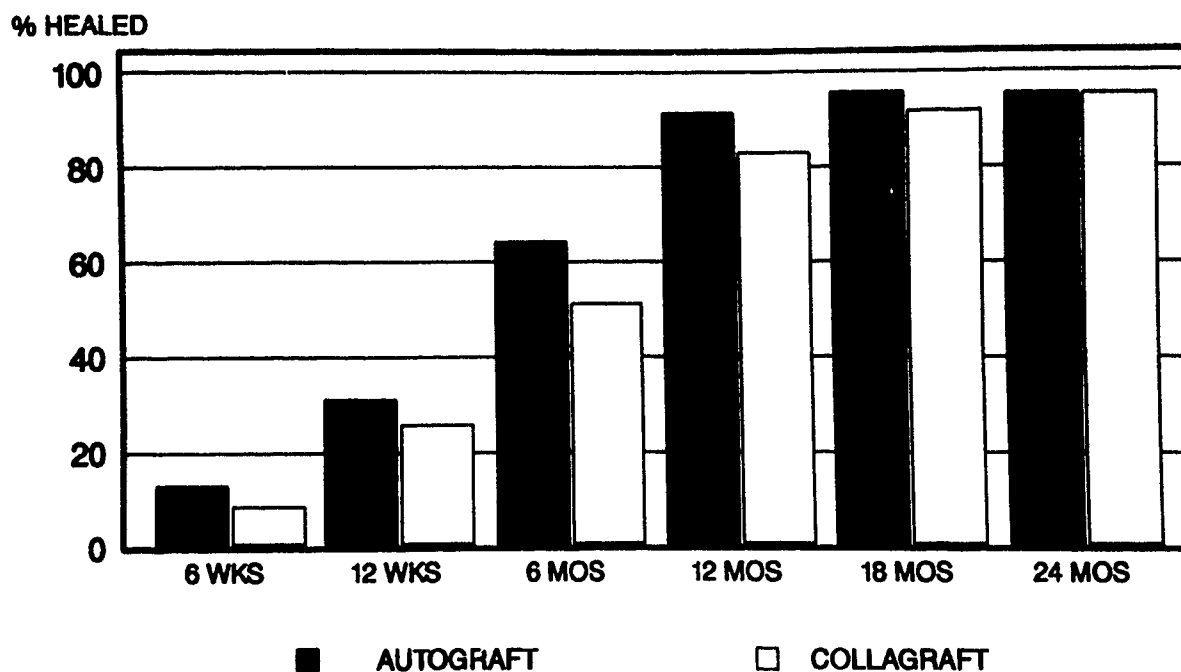
Figure 3

PATIENT DEMOGRAPHICS

	No. of patients	No. of FX	Sex (M/F)	Mean age (yr)	No. of closed FX	No. of open FX
Control	128	150	88/40	36 (18 - 72)	107 (71.3%)	43 (28.7%)
Collagraft	139	161	102/37	39 (18 - 70)	108 (67.1%)	53 (32.9%)

Table 1

Collagraft bone graft substitute clinical study: complication profile

	Collagraft n	Collagraft %	Autograft n	Autograft %
Fracture site infection	9	5.7	13	8.7
Iliac crest infection	0	0.0	2	1.3
Loss of fixation	6	3.8	2	1.3
Hematoma	1	0.6	0	0.0
Delayed union	3	1.9	4	2.7
Nonunion	4	2.5	3	2.0
Wound drainage (fracture site)	1	0.6	2	1.3
Wound drainage (iliac crest)	0	0.0	3	2.0
Wound dehiscence	1	0.6	0	0.0
Deformity	5	3.1	5	3.3
Pain due to fixation device	1	0.6	1	0.7
Loose hardware	1	0.6	1	0.7
Refracture at plate removal	1	0.6	1	0.7
Total	33	20.6	36	24.0

Total number of collagraft bone substitute fractures = 161; total number of autogenous fractures = 150

Table 2

GROUP	PRIMARY DEBRIDEMENT CFU/gr	SECONDARY DEBRIDEMENT CFU/gr
1	5.89×10^6	6.7×10^2
2	3.16×10^6	1.95×10^7
3	2.04×10^6	1.10×10^7
4	8.13×10^6	3.63×10^6

Table 3

BIOLOGIC AND IMMUNOLOGIC ASPECTS OF BONE GRAFTS

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INTRODUCTION

The need to transplant bone and articular surfaces for repair or replacement of skeletal deficits has been recognized for centuries. Our current approach to these clinical challenges, however, was initiated by the observations of Ollier[1] in the mid-19th century and the surgical experiences reported by Macewan in 1881.[2] The use of massive osteochondral allografts began with Lexer,[3] but the major scientific and technical innovations responsible for our contemporary success have been much more recent. Parrish,[4] in the United States, and Volkov,[5] in the Soviet Union, developed sound operative approaches and principles with encouraging clinical results. Burwell,[6] around this same time period, began a comprehensive and systematic investigation of bone allograft biology, with particular emphasis on the influence of preservation techniques and the consequences of immune responses presumed to be an integral aspect of the fate of these grafts. Similarly, Curtiss, Herndon, and colleagues[7,8] identified the ability of deep-freezing to decrease the immunogenicity of bone allografts, an observation that simultaneously laid the foundation for modern bone banking methodology. Mankin and co-workers[9] further developed these biologic and clinical fundamentals into a more mature approach applicable to limb-sparing reconstruction of large skeletal defects following tumor resection.

While this experience and that of many others, including treatment of traumatic injuries[10] and the restoration of lost bone stock encountered in revision total joint arthroplasty,[11] has been encouraging, the overall failure rate of approximately 25-30% for massive allografts[10] has belied complacency. The reasons for failure are in some cases understood,[12] but too often unpredictable and enigmatic. One logical source of explanation for allograft compromise is the consequence of immune responses or immunologic rejection. Clarification of the role of immunocompetent cells and their

products in the fate of osteochondral allografts has been the topic of investigation for more than 40 years. [6,13-15] Despite this lengthy effort and enormous improvements in technology, the nature of responses evoked and their biologic impact remains unclear and, perhaps, controversial. Results of outcome vary among animal models and, more importantly, the extrapolation of animal-derived data to humans has not always been convincing. Nonetheless, it is clear that the clinical fate of bone and cartilage allografts reflects the biology of repair, remodeling and incorporation and that, to some degree, this biology is influenced by immunocompetent cells and their molecular products.

BIOLOGY

The ability of bone as a tissue to maintain its integrity, repair when fractured or incorporate as a graft is based upon its unique capacity to regenerate. The reparative process throughout the rest of the body results in scar formation, but bone is usually repaired or replaced by additional osseous tissue. In terms of homeostasis, this sequence of important biologic events has been termed the remodeling cycle.[16-18] Surface activity on a bony trabeculum shifts from a resting or inactive status to one of activation. Signals responsible for this activity appear to be several in number, probably sequential or cascading in nature and originate from both cells and matrix. The first population of cells recruited and activated are osteoclastic and result in removal of a portion of preexisting matrix. These large multinucleated giant cells isolate a portion of bone beneath their cell membrane and then actively manipulate this microenvironment by active ion exchange and enzyme release to resorb a scalloped divot in the original bony surface. Osteoclasts disappear as the bone enters a reversal phase prior to the recruitment and activation of osteoblasts. These bone-forming cells then lay down a seam of osteoid which is subsequently mineralized. Some osteoblasts become engulfed in this new bony matrix and persist as osteocytes, mononuclear cells responsible for maintaining their local calcified matrix environment. This completes the sequence of events representing the remodeling cycle, also the end point of fracture repair and graft incorporation.

The histologic stages of bone graft incorporation have been described by numerous investigators over the past several decades, with little change or controversy.[19-21] The physiologic mechanisms that control these activities, however, remain incomplete and

poorly understood.[17,22] The initial stage of bone graft incorporation, for grafts not immediately revascularized by surgical techniques, includes hemorrhage, necrosis of implanted osseous tissue and some degree of inflammation. Unless the blood supply to the transplanted segment is quickly reestablished through vascular reanastomosis, only those cells very close to a bony surface can survive by diffusion. As the hematoma surrounding the graft begins to organize, the graft itself becomes engulfed in a fibrovascular tissue. The remainder of events reflect a partnership between the graft and its host bed; both sources of contributors being crucial to the success of the process.

Graft contributions to incorporation include a very small percentage of surviving cells, osteoconduction and osteoinduction. Regardless of a loss of cell viability, the bony matrix can function in a passive mode as a trellis or scaffold for the ingrowth of neovascularization and new-bone formation, a process termed osteoconduction. Osteoinduction is the active phenomenon created by release of humoral signals presumably from the matrix alone which stimulate revascularization and recruitment of differentiated cell populations.[16,22,23] Bone morphogentic protein (BMP) is one of the best known, yet still elusive, matrix-derived signals for osteoinduction.[24]

The host bed contributes all of the new blood vessels as well as the vast majority of cells required to resorb and replace bone. The fibrovascular stroma finds its way through preexisting haversian and Volkmann canals in the case of cortical grafts or percolates through the medullary spaces of cancellous bone. In the cortex, this neovascularization is augmented by osteoclastic activity that widens preexisting canals and removes nonviable matrix in the process. This results in a substantial increase in porosity over baseline, and for this reason cortical bone graft becomes transiently and predictably weak until osteoblasts become active. In cortex this is demonstrated by filling in of the enlarged haversian canals to create osteons. In cancellous bone, preexisting, dead trabeculae become sandwiched between layers of osteoid and newly mineralized bone.

With time, both cortical and cancellous bone go on to enter the remodeling cycle. Because of its looser structural anatomy, cancellous bone is revascularized sooner than cortical bone, and the process of incorporation (resorption and replacement) tends to be more complete.[19]

This description of events is the same qualitatively for autogenous bone as well as allogeneic bone grafts.[19,20] Allografts are quantitatively inferior in terms of both rate

and extent of replacement with new bone. To a degree, these quantitative differences vary with the manner in which bone allograft is prepared and the extent of genetic disparity. The majority of available data in this regard are qualitative in nature.

CLINICAL IMPLICATIONS OF BIOLOGIC EVENTS

Since bone graft incorporation requires the participation of both the graft material and its host bed, alterations in either the transplant or the recipient may have substantial impact upon the repair process.[12] Local changes in the host bed that might detract from graft incorporation include the presence of a foreign body or other material interposed between the graft and its recipient bed (e.g., polymethylmethacrylate), infection, necrotic soft tissue or tissues compromised by treatment such as irradiation or a bony bed consumed by a neoplastic or metabolic abnormality. Indeed, any circumstance that interferes with the ability of the host bed to generate new blood vessels or to recruit and convey to the graft cell populations capable of resorbing and forming bone will ultimately compromise graft incorporation.

There are also systemic influences that detract from graft incorporation, primarily by reducing or injuring the pool of differentiated cell populations necessary for the process. Chemotherapy, for example, can substantially reduce the number osteoprogenitor cells available to the graft.[25] There is also some indication that nonsteroidal anti-inflammatory drugs, particularly indomethacin, also suppress new-bone formation, and perhaps certain forms of immunosuppression, such as Cyclosporin–A, in selected doses may also be adverse.[26]

IMMUNOLOGY

There are numerous studies in a variety of animal models that demonstrate several immunogenic components of bone. This includes, to a lesser degree, collagen and matrix, but clearly cell surface antigens are the most potent source of immunogenicity.[14] The major source of cells in bone resides in the marrow, and a multitude of hematopoietic cells all share common histocompatibility antigens which are the primary source of immunogenicity in terms of transplantation. This activity is clearly reduced by deep-freezing and reduced even further, to the point of often being nondetectable, by freeze-drying. Again, these phenomena have been documented in a

307

wide variety of laboratory models and following numerous approaches to bone graft preservation.

Two particular questions remain perplexing with respect to bone allograft antigenicity, particularly in humans. First, it is difficult to define "immunologic rejection" of a bone graft. Markers of biologic activity are scant, particularly if noninvasive techniques are preferred. Second, all immune responses are not equally detrimental, in fact it is possible that some patterns of sensitization are actually protective or beneficial to bone graft incorporation. These circumstances are just beginning to become apparent. Preliminary data from 74 patients receiving massive frozen osteochondral allografts, in the course of a multi-institutional study, would suggest that sensitization to class II histocompatibility antigens, and probably class I as well, is associated with a reduced rate of clinical success.[27,28] However, it is equally clear that sensitization did not predictably preclude a satisfactory clinical outcome. While 20 of 22 (91%) patients who failed to become sensitized following receipt of allografts had an excellent or good clinical result, 65% of the 52 patients who were sensitized also did well by the same clinical criteria. The sensitized and nonsensitized groups were statistically significantly different in their overall rate of success, but a satisfactory result was the norm in both circumstances. Consequently, a meaningful correlation between immune responses and graft biology in humans remains elusive.

Other fascinating implications involving the imm゛ system and bone remodeling revolves around the fact that both processes involve cells of common ancestry and it is becoming clear that cells of the immune system play an active role in regulating bone remodeling.[29]

BANKING METHODOLOGY

The clinical use of bone allografts also requires implementation of appropriate bone banking approaches. These methods must assure both safety and efficacy, and guidelines have been developed by the American Association of Tissue Banks to address these major concerns.[30,31]

There has been an evolution in the consent laws used in this country. Until recently, both tissue and organ donation were based upon individuals or their family voluntarily identifying themselves as possible donors or dependence upon the ad hoc

activity of transplant coordinators searching out potential sources of these transplantable resources.[32] An important variation of this voluntary consent approach has been the principle of require request or routine inquiry. In this fashion, health care institutions are compelled to develop programs for identifying potential donors and creating a system by which these patients or their appropriate next-of-kin are asked whether or not they would like to participate in the donation process. The choice remains that of the individual, but the institutions, by law, now have the burden of establishing a programmatic approach that raises the question on a routine basis.

Donor selection remains a very crucial step in bone banking. Potential donors must be screened by virtue of their past medical history, circumstances surrounding death in the case of cadaveric sources and the use of a variety of laboratory tests, all designed to eliminate individuals with potentially harmful transmissible diseases or disorders that might adversely impact upon the biologic properties of the tissue to be recovered. It is particularly important to eliminate tissues that might be contaminated by infectious diseases, including bacterial, fungal and viral; and both the past medical history and cultures of each tissue recovered are important controls. Similarly, venereal disease, hepatitis and AIDS can each be screened for utilizing standard tests. In the case of femoral head donation at the time of total joint arthroplasty, it is the recommendation of both the Centers for Disease Control and the American Association of Tissue Banks that a second test be accomplished approximately 90-180 days following donation to reflect the window during which antibodies to the HIV virus may be generated.[33,34] Other areas of concern and contraindication include the presence of cancer, metabolic bone diseases, diffuse collagen disorders, a history of slow virus disease or any systemic disorder of unknown etiology as well as the presence of toxic substances in potentially harmful amounts.

Tissues may be recovered in a clean environment and depend upon secondary sterilization. Alternatively, tissue may be recovered in an operating room environment and kept sterile throughout the recovery and packaging procedures. Secondary sterilants include the use of ethylene oxide or high-dose irradiation followed by cultures to attest to the sterility of each specimen. It is crucial to be certain that secondary sterilants are effective, do not produce any biologic or biomechanical changes that interfere with the intended use of the graft material and that no toxicity is imparted to

the graft prior to its implantation. It appears that the HIV virus is susceptible to doses as low as 0.5 megarad of irradiation when isolated in vitro, but even megarad doses have not proven reliable for sterilization of HIV infected bone.[35,36] Most bacteria require 1 to 3 megarads for sterilization by irradiation. Doses above 3 megarads are associated with changes in biologic and biomechanical properties that interfere with the usefulness of these transplantable tissues.[37]

It is the author's preference to acquire tissues sterilely in an operating room, confirm their lack of contamination using standard culture technique, preserve tissues for long-term storage by deep-freezing and then packing these grafts in sterile containers or wraps for storage at -70 to -80°C. This approach is compatible with cryopreservation of articular cartilage[38] and suitable for storage for several years without apparent change in the biologic or biomechanical properties of the tissue.[39] Freeze-drying requires additional specialized equipment, but results in tissues that may be stored indefinitely in sealed, evacuated containers at room temperature. Such an approach is not, however, compatible with cryopreservation of articular cartilage. In addition, lyophilization may change the biomechanical properties of bone.[39]

At the present time, large osteochondral allografts are chosen based upon size and fit, and not upon immunologic parameters.

CONCLUSIONS

Bone grafts, autogenous or allogeneic, undergo a predictable sequence of biologic events. Circumstances important to graft incorporation include contributions both by the graft itself as well as the host bed. Consequently, there are factors that alter the success of bone graft procedures and predictably predispose to failure. Biologic and immunologic properties of bone grafts may be altered as a consequence of graft preservation techniques. Immune responses directed against cell surface antigens of cells found in bone occur. The consequences of these responses on bone biology are unclear, but remain a topic of active investigation. Knowledge of normal graft biology, changes imparted during banking methodology and the nature of the host bed all contribute to improved clinical results. Indeed, the basis of clinical success is rooted in knowledge of fundamental biologic principles.

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AIDS AND BONE GRAFTS: RECOMMENDATIONS

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INTRODUCTION

The first person reported to the Centers for Disease Control (CDC) with transplantation–associated Acquired Immunodeficiency Syndrome (AIDS) was a bone transplant recipient. Previous reports had identified transmission of human immunodeficiency virus (HIV–I) in the transplantation of kidney, liver, heart, pancreas and possibly skin and semen. In 1988, however, at the time of the reported case of AIDS following a bone transplant, none of the infected recipients of these organs or tissues had apparently developed AIDS. The case report on bone clearly shows that the transmission of AIDS is a risk in bone transplantation. A review of the case and an analysis of the risks involved in the use of musculoskeletal tissue transplants provides a foundation for recommendations for safe bone banking and musculoskeletal allograft uses.

Case Report (Synopsis – MMWR October 7, 1988)

In February, 1988, a bone transplant recipient was diagnosed with AIDS.[1] The recipient was a young woman who in November, 1984 received a femoral head allograft from a bone bank in a hospital in which she underwent a spinal fusion for idiopathic scoliosis. The graft had been obtained from a donor who had a total hip arthroplasty twenty–four days prior to her surgery. The bone was stored frozen a -80° C and was not opened between the time it was placed into the bone bank freezer and removed for clinical transplantation.

The patient was a health care worker who washed gynecologic speculae without using gloves, but she denied a needle stick or mucous membrane exposure to blood or other body secretions. She received no blood transfusions during her surgery. She had

been married for four years prior to her surgery, and she denied sexual partners outside her marriage. Her husband also denied extramarital sex partners and any other risks for HIV infection. He was tested for HIV antibody in February and again in April of 1988 and both tests were negative.

The patient had a prodomal syndrome for a viral infection about three weeks after her surgery. She became asymptomatic thereafter until approximately two years postoperatively when she noted enlarged axillary lymph nodes on a breast self-examination. A physician confirmed this finding at that time and at a second examination six months later but performed no further diagnostic procedures.

At approximately 3 1/2 years post-transplant, the patient returned to her physician with complaints of fever, a non-productive cough, malaise and generalized chest pain for two weeks. Physical examination showed oral and vaginal candidiasis and generalized lymphadenopathy. She was found to be positive on blood testing for HIV antibody and subsequently diagnosed with pneumocystis carinii pneumonia (PCP) and AIDS. The patient's illness initially improved with therapy, but she has apparently subsequently died.

An analysis of the past medical history of the donor showed that he had an excisional biopsy of a cervical lymph node approximately five months prior to his hip arthroplasty. Pathologic analysis of the node showed non-specific hyperplasia. At the time of femoral head donation, his physical examination noted an enlarged lymph node in the right cervical area. Approximately 1 1/2 years after bone donation, the donor developed PCP, was found to be positive for HIV antibody, and was diagnosed as having AIDS. At the time of diagnosis, the donor reported previous intravenous drug use but denied other risks for HIV antibody. The donor's wife was also found to be positive for HIV antibody. The donor died of PCP and atypical mycobacteriosis approximately 2 1/2 years after bone donation.

Following this report, the Public Health Service reiterated its recommendation that all donors of human allografts be evaluated for risks associated with HIV infection and be tested for HIV antibody. A special conference, which included representatives of the CDC, Food and Drug Administration (FDA), American Academy of Orthopaedic Surgeons (AAOS) and the American Association of Tissue Banks (AATB) was held in Atlanta in August, 1988. This group discussed draft recommendations for the preven-

tion of HIV transmission by bone transplantation. Based on this meeting and prior investigations and publications,[2] the Public Health Service recommended the following measures to prevent the transmission of HIV in tissues.

1) A review of the donor's medical record, testing the donor for HIV antibody and interviewing live donors for risks for HIV infection should be performed.

2) Responses to interview questions should be recorded on a form that is signed by the donor to acknowledge that he or she has read the recorded responses and that they are correct. The completed form should be kept in the tissue blood bank.

3) Bone obtained from living donors should be quarantined for 90 days. Living bone donors should be re–tested for HIV antibody at least 90 days after donation and surgical bone from living donors who are negative for HIV antibody on this repeat test should be distributed for transplantation.

4) Bone from donors who are not available for re–testing, including cadaveric donors, may be used when bone from re–tested living donors is not available and is not appropriate for use.

RECOMMENDATIONS

Transmission of the AIDS virus in a musculoskeletal allograft is primarily related to the probability of transplantation of blood in the graft. The target cell for HIV–I is the CD–4 positive T lymphocyte. This cell is a thymus–derived cell and is found in the peripheral blood as well as in the marrow. Any bone graft that transplants blood or blood products, such as bone marrow, might also transplant the AIDS virus. Consequently, one of the most important recommendations for the avoidance of AIDS transmission in a human allograft is to use a graft which does not contain blood or blood products.

The transmission of blood in a musculoskeletal allograft may be avoided by washing the blood out of the tissue. This procedure is frequently performed in processing bone grafts which are cut or powdered and freeze–dried for storage. Although bone grafts may not be guaranteed to be completely free of virus because it is difficult to wash all the blood out of a bone, the probability of survival of virus in processing, which in most tissue banks involves frequent washes of the bone and antibiotics or alcohol soaking, is undoubtedly extremely low. Therefore, one method

which may successfully reduce the probability of viral transmission in a bone graft is to use a processed graft whenever possible.

For fusions, treatment of fractures and cysts, processed tissues, such as freeze–dried cancellous chips, perform satisfactorily. Other applications of these types of tissues include the treatment of bone loss and fractures in failed joint replacements. However, processing including freeze–drying, affects the biomechanical strength of certain types of musculoskeletal allografts such as whole segments of long bones.[3] In addition, because these types of grafts, such as osteoarticular grafts, retain marrow and marrow elements because they include the metaphyseal and epiphyseal areas of long bones, they obviously also must contain blood. The tissue bank must therefore rely on a method other than removal of blood and processing to lower the probability of viral transmission when providing these types of grafts.

Currently, the most common method of decreasing the probability of AIDS transmission is by testing the donor for evidence of HIV–I. The most frequently used test for this purpose is an enzyme linked immunosorbent assay or ELISA which detects antibody to HIV–I. By exposing a patient's serum to antigens of the AIDS virus, such as proteins that coat the virus, antibody present in the serum of the person being screened will bind to the antigens and are detected by the assay.

The ELISA test is extremely sensitive. If antibody is present in the test serum, the test has about a 99% chance of detecting the antibody. However, the amount of antibody present must be sufficient to produce a change in the colorimetric pattern used as a standard to determine a positive test. If the antibody response in the person being tested has been inadequate to produce an antibody level that is detectable, then the ELISA test may not be positive. Based on analysis of the blood donor population, the probability of obtaining a bone graft from an AIDS infected donor who has a false–negative ELISA ranges from 1:40,000[4] to 1:153,000[5].

A false–negative ELISA may occur in patients who are immunocompromised or anergic and do not produce antibody to the virus or in patients who have very recently been exposed to the virus and have not produced a sufficient amount of antibody to provide a positive test. Imagawa reported on a group of the former type of patients from San Francisco who had negative ELISA antibody tests but were found to be infected by the AIDS virus by polymerase chain reaction (PCR) testing.[6] In 1988, Ward reported on

317

a group of patients of the latter type in his study of blood donors who had negative ELISA tests but who transmitted the AIDS virus to people who received their blood in transfusions.[4] Therefore, it is clear that there are rare instances in which the ELISA test may be falsely negative. The current generation of assays is extremely sensitive and reliable, however, and may prevent recurrences of the above types of problems.

To supplement and strengthen the screening value of the ELISA test, several methods have been used. For living donors, the allograft may be quarantined for a period of time and the donor re-tested. According to CDC data, re-testing allows time for antibody development. A six month quarantine of living donor bone has been recommended by the AATB. Second, in addition to delayed blood testing, the donor should be carefully screened by history and physical exam according to CDC and blood donor guidelines. This procedure is relatively easy when screening living donors. For cadaver donors, however, family knowledge of the medical history of the donor is frequently incomplete or inaccurate.

A third method of screening has been the use of the AIDS antigen test. This test was developed after the ELISA antibody screen and was originally believed to provide more accuracy in screening for the AIDS virus than the antibody test. However, Alter showed that in one half-million blood donors who were screened by both the antibody and the antigen tests, no donor who had a positive antigen test did not have a positive antibody test.[7] Thus, the value of the antigen test as a more reliable screening test than the ELISA antibody test is questionable. The majority of tissue banks now screen only for antibody.

A fourth method of assuring the safety of a bone graft is to sterilize the graft. The two most popular methods of sterilization for musculoskeletal grafts are ethylene oxide (ETO) and irradiation. Both methods have been shown to kill the AIDS or similar retrovirus.[8,9] However, both methods also have adverse effects. By products of ETO may be retained in tissues and produce inflammation in the recipient of the graft.[10] Likewise, irradiation may produce biomechanical weakening of a large bone graft.[11] Thus, sterilization of a bone graft is not an ideal method of preparation or processing.

SUMMARY

No human allograft may be completely guaranteed free of virus. However, by screening donors by history, physical examination and blood tests, the probability of

recovering tissues from a viral carrier can be reduced to extremely safe levels. By using bones which have been washed free of blood, the probability of transplanting the virus in the tissues is reduced to a very safe level. By quarantining tissues and testing the donor at a later date, or irradiating the tissues, the probability of transmission may be lowered even further. Given this knowledge, the surgeon can now tell recipients of allografts that although there is still a small risk in using allografts that although there is still a small risk in using allograft tissues, the risk has been reduced to safe levels.

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BONE GRAFTS AND ALLOPLASTIC MATERIALS
IN NEUROSURGERY

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INTRODUCTION

Two years have passed since the first Bone Symposium sponsored by Walter Reed Army Medical Center at the uniformed Services University of the Health Sciences in Bethesda. In that as in this convocation, I have been invited to address the use of bone grafts and alloplastic materials as they are applied in neurosurgery. Whereas, in the first meeting I discussed cranial applications, today I will review neurosurgical experiences with grafts in spinal operations.

Although animal investigations in our laboratory have centered on cranial healing, over the past fifteen years we have had a far greater experience in clinical studies of the repair of allogeneic bone in spinal fusion. Over 30,000 human bone allografts from our tissue bank have been prepared since 1976 for human implantation. Most have been used for achieving spinal stabiliztion Themes of this paper include the biology and repair of allogeneic bone in human cervical and lumbar interbody fusions and a quantitative analysis of the clinical result from the use of these allografts.

A full appreciation of the importance of the spinal column in clinical neurosurgery is recent. The prevailing and traditional mindset in neurosurgical training programs has focused 60% of a resident's time on cranial and 40% on spinal conditions. In clinical practice, the exact opposite occurs; neurosurgeons in communities allocate about 60% of their time to spinal disorders. The incontrovertible pioneer among neurosurgeons in the advocay and practice of spinal fusion is Doctor Ralph Cloward. He encountered derision in 1947 at the Harvey Cushing Society Meeting when he presented his first 100 cases of posterior lumbar interbody fusions. A respected discussant from Virginia denounced his presentation to the pleasure of the audience: "We are neurosurgeons and as such should confine our activities to the trephine and the rongeur and leave the

hammer and chisel to the orthopedic surgeons."[1] Cloward rejected that advice and over the past half century has instructed his colleagues in his spinal fusion techniques of lumbar and cervical spine fusion.

TYPES OF SPINE FUSIONS

The superiority of autogeneic bone in obtaining fusions has been extraordinarily well documented in research animals and remains the graft of choice in posterolateral spine fusions with or without instrumentation. Viable cells and undenatured collagen can be transferred directly to a recipient bed at the lateral facet, pedicle, transverse process confluence. First order autograft must be used in the posterolateral locations because there the blood supply is suboptimal, decorticated bone surfaces are exiguous, compressive forces at the graft/host interfaces are tenuous and are supplied only by overlying paraspinous muscles. Allogeneic cancellous bone chips can be admixed with fresh autogeneic, marrow-containing bone to augment bone mass (a composite graft)[2] expecially when the goal is arthrodesis of multiple motion segments in a small or young patient. Even in the more favorable interbody recipient bed, Doctor Paul Lin strongly advocates autogeneic over allogeneic bone.[3]

Cloward was the first to use allografts routinely in interbody applications after Inclan's seminal publication on bone banking in 1942.[1] Fusion rates in lumbar and cervical interbody fusion with allogeneic bone can be identical with that achieved with autogeneic bone. At least eight complications have been described at the usual prime donor sites for autografts.[4] The avoidance of these (especially donor-site pain) associated with the acknowledged benefits of allogeneic bone from a suitable anatomical donor has persuaded many neurosurgeons to use banked bone. These advantages include the following: 1) decreased operation time; 2) stronger corticocancellous bone from young donors; 3) availability of multiple shapes and sizes that may be difficult to obtain from the patient's store without risk of fracture, deformity, pain, blood loss, etc. All of these factors for many spines surgeons exceed the perceived major disadvantages of allogeneic bone: 1) absence of gentically identical living cellular and protein substrates; 2) the need to sterilize bone allografts; 3) the adverse effects on inductive proteins by processing techniques; 4) the biomechanical reductions in strength of allografts by freezing or freeze drying for storage or radiation for sterilization; 5) the potential

transmission of disease; 6) the potential for residual chemicals in grafts that have been sterilized; 7) time delay in incorporation/remodeling of the allograft compared to the autograft. Allografts less than optimally treated can instantly collapse, resorb, or result in nonunion and suffer delayed fatigue fracture.

Alloplastic materials such as methylmethacrylate are used most often in spinal fusion when longevity is limited.[5] A combination of acrylic incorporating posterior or anterior bone surfaces bound together by wires, rods or plates provides immediate stability that is desirable in the elderly patient or one with malignancy. Because the plastic is an inert foreign body without potential for revitalization long term, it is vulnerable to fracture under load, infection or failure. Ceramic implants composed of coralline hydroxyapatite or tricalcium phosphate are biocompatible, incorporate, but are brittle, easily fracture and have 55% of the strength of cancellous bone.[6,7]

ADVANTAGES OF INTERBODY LOCUS FOR SPINAL STABILIZATION

The interbody location is superior to posterolateral region in achieving immediate spinal stability when one considers both biomechanical and biological factors. The task of defining an incompetent disc or unstable motion segment is problematic. The disc bears eighty percent of the load at the motion segment. Only two forms of mechanical intervertebral joint failure have been identified: those due to axial compression and those due to axial torsion.[8] Mechanical failure occurs when the spine buckles under high axial loads: axial compression in the sagittal plane or axial torsion in the nonsagittal plane (lateral bending with flexion/extension). In axial compression the vertebral end plate fractures first with the annulus and vertebral body thereafter taking up the load. By contrast in torsional injuries there is no back-up mechanism. The joint becomes potentially unstable and regains strength by healing and scar formation. The annulus is the most important structure to resist torsion. Facets resist torsion to a lessor extent, but torsion deformation curves for the disc and whole joint are the same.

A degraded intervertebral disc that has suffered torsional injury and lost its resistance to compressive loading is the hallmark of mechanical instability. *Ipso facto* its replacement by compatible bone grafts is essential to regaining stability, such that loads normally tolerated will not result in excessive or abnormal spinal motions, displacements or strains, or cause progressive deformity or neurological injury.

Moreover, the instantaneous axis of rotation lies in the disc space.[9] From beam mechanics the judicious placement of distraction blocks symmetrically about the instantaneous axis of rotation of the motion segment provides immediate resistance to displacement (namely rigidity) by neutrally balancing this pivot point. Rolander demonstrated motion at the disc space even after a successful posterolateral fusion; others have shown symptoms can occur from this motion. Because of the immediate stability obtained by distractive interbody bone blocks under compressive loads, instrumentation with "heavy metal" is obviated.

The most important biological factor for achieving fusion is a highly vascular, marrow-rich host bed adjacent to the graft that provides a rich source of osteoprogenitor cells. Strong compressive forces at the host/graft interface further promote fusion. The largest articulating surface areas between vertebrae are the adjacent bodies. Rapid revascularization of cancellous bone of the graft can be achieved, thus preventing major resorption.

CERVICAL ANTERIOR INTERBODY FUSION

Anterior cervical fusions were first done by Cloward and Smith and Robinson. In the Cloward procedure, the disc is completely removed. Drills of various diameter (10mm, 12mm, 14mm, 16mm) are used to penetrate adjacent vertebrae leaving a posterior cortical shelf of bone. The cortical end plates are removed, exposing the cancellous bone of the vertebrae to the cancellous bone of the cylindrical allograft that is impacted into the interbody space.

In the late 1970's, the experiences of 14 neurosurgeons were collated.[10] In 115 patients fusions occurred by x-ray criteria in 160 interspaces out of 166 (96%). This fusion rate is identical with autogeneic bone, though usually takes about four months rather than three.

Allogeneic fibular struts can be used to span multiple cervical vertebrae with an extremely high fusion rate. Others use tricortical grafts of horseshoe shape in performing the Smith-Robinson technique of fushion.

POSTERIOR LUMBAR INTERBODY FUSIONS

In the posterior approach to interbody fusions, a total posterior and anterior decompression of nerve roots and the cauda equina is a necessary precondition for the

preparation of a receptive rectangular interbody space for interposition of grafts. A laminotomy, medial facetectomy, superior medial pediculotomy and total disectomy remove all central and far lateral sources of nerve root impingement concomitant with allowing lateral access to an interbody space entirely denuded of disc and cortical end plates. Grafts placed adjacent to the anterior longitudinal ligament and entirely mortised within the space carefully crafted to receive them maintain height of adjacent motion segments, thus preventing foraminal stenosis. A spectrum of available allograft sizes eliminates the problem of poor fit: poor contact at graft/host interface will prevent osteoconductive remodeling by "creeping substitution". Vessels and mesenchymal/osteoprogenitor cells do not jump across spaces. Total fill of the interbody location with allograft eliminates any tendency of grafts to angle under rotational stresses and then to retropulse back into the canal. My technique in the posterior lumbar interbody fusion follows the teaching of Cloward.

Tricortical allografts from decedents who meet rigid criteria for donorship were used in this series. All grafts were sterilized with 100% ethylene oxide and then lyophilized according to a protocol developed in the 1970's.[11] Prior to placement in the interspace, grafts were hydrated under a vacuum.

ANALYSIS OF OUTCOME

Criteria for evaluating the results of treating lumbar spine disorders vary widely. In an effort to encourage uniformity in the interpretation of outcomes from various treatments regimens, we proposed an anatomic-economic functional scale in 1985 that can be easily applied, is reproducible and has little inherent tendentious observer subjectivity.[12] This scale was modified after Urist and Dawson who refer to the Massachusetts General Hospital Grading System in their evaluation of posterolateral fusions with autogeneic and allogeneic bone.

An anatomic scale depicts results according to various permutations of the adequacy of decompression and degree of stability of the lumbar spine. An economic scale reflects graded performance in the competitive market place or when appropriate in school, domestic or retirement activites. A functional scale represents levels of activity based upon the extent of pain and neurological impairment. Together these three scales

quantitatively express the full spectrum of responses to treatment. They may be applied to patients before and after treatment to record the extent treatment affects the population of patients studied (Table 1).

Anatomical fusion rate was high. The ten-point scale combining economic (five points) and functional (five points) outcomes was applied to patients before and after PLIF (posterior lumbar interbody fusion). An ordinal scale was then used to measure results: Excellent (9,10), good (7,8) fair (5,6), poor (2-4).

PATIENT SERIES

Our experience consists of 234 operations on patients who have undergone PLIF at one or more levels. The first 105 patients will be analyzed in detail; results from the second group of over 100 patients appear comparable and are under current evaluation (Table 2).

Sixty males and forty-five females had an average age of 43 years. Entry level physical stresses to their lower backs were equally divided between heavy, medium and light. About 60% were indemnified by Workman's Compensation Insurance; 40% had private insurance. PLIF of L4-5 was performed twice as often as at L5-S1 (68:33). A histogram of patient's age by decade of life shows a range from the second to the ninth with most clustering between 30 and 60 years. (Table 3).

Indications for fusion encompassed conditions resulting in an incompetent lumbar disc (Table 4). Stability of motion segment was determined after one year with anteroposterior and lateral bending x-rays in all patients. Computed axial tomographic scans across motion segments with coronal reconstructions were used in some to assess trabecular bone between host bone and allografts. Fusion occurred in 102 of the 105 patients (97%) (Table 5). Preoperatively, the combined economic/functional scales averaged 3.3: one year after operation the average economic/functional scale was 8 (a gain of 4.7) (Tables 2,6). Eight-four percent of the patients received an excellent or good result using specific criteria; 16% were unsuccessful (fair or poor).

Thirteen patients had isthmic or degenerative spondylolisthesis; all fused (Table 7). All 17 patients with numerical values 6 and below (fair/poor) were covered by industrial insurance. Among the 22 patients with the "failed back syndrome" (characterized by one or more previous operations at that level) 72% achieved excellent/

good results (Table 8). Five of eight patients having previous chemonucleolysis had favorable results (63%).

Seven complications occurred among these 105 patients (Table 9). All were transient. Notably absent in the first 105 patients and the entire series of 233 operations were death, retropulsion of bone blocks or infections.

Attention must be directed to the higher success of decompression/fusion (97%) compared to the clinical success rate (84%). Numerous authors have noted this disparity between anatomical and clinical outcome.

BIOLOGY OF INTERBODY FUSION

With the high fusion/low pseudarthrosis rates only a few observations in three patients about the microscopic appearance of allograft remodeling are permissible. In two patients, the allografts remodeled from the upper vertebrae, but failed to do so from the lower vertebrae. Islands of new bone interdigitate with necrotic bone of the graft. At the site of nonunion newly formed cartilage surrounds necrotic bone. Areas of cartilaginous metaplasia possibly reflect some combination of poor fit, impared blood supply, low oxygen tension. They are potentially transformable to new bone.

Two years ago the absence of osteoclasts in remodeling cranium was emphasized. In the human spine as in the cranium, osteoclasts are very rarely noted in some and never in other areas of remodeling.

SUMMARY

Cervical and lumbar interbody fusions can successfully be accomplished with allogeneic bone that is sterilized with ethylene oxide and freeze dried. Allografts are remodeled by osteoconductive "creeping substitution" from adjacent marrow-rich vertebral bodies. Fusion rates of over 95% in cervical and lumbar interbody locations compare favorable with autografts. In the lumbar interbody fusion a high clinical success rate (84%) defined by strict criteria can be achieved. Scrupulous adherence to highest known surgical and bone banking standards equally contribute to the high fusion/clinical success rates, low transient complication rate and total absence of infections or retropulsion of allografts in 234 operations.

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TABLE 1 – COMBINED ECONOMIC / FUNCTIONAL
AND ANATOMIC RATING

RATING	EFA (TOTAL) SCORE			
Excellent	15,	14,	13	
Good	12,	11,	10	
Fair	9,	8,	7	
Poor	6,	5,	4,	3

TABLE 2 – PATIENT POPULATION (105)

	FEMALES	MALES	BOTH
Number of patients	45	60	105
Age at operation	44.6	42.0	43.1
Pre-operation E/F Score*	3.2	3.4	3.3
One-year E/F Score	8.3	7.8	8.0
Gain in E/F Score	5.1	4.4	4.7
Stress: High	4	33	37
Medium	25	11	36
Low	17	16	32
Insurance: Private	28	15	43
Wkmn's Comp	17	45	62
Levels fused: L2-L3	0	2	3
L3-L4	4	4	8
L4-L5	3	35	68
L5-S1	10	23	33

*E/F Score is the combined score of the economic and function evaluations

330

TABLE 3 – PATIENT POPULATION BY DECADE OF LIFE

DECADE	INCIDENCE
10 - 19	1
20 - 20	14
30 - 39	27
40 - 49	31
50 - 59	20
60 - 69	11

TABLE 4 – INDICATIONS FOR LUMBAR FUSION

A. Instability

1. Spondylolisthesis

2. Subluxation on the bending film > 4mm

3. Chronic arthritic changes

4. Previous operation(s)

5. Previous chemonucleolysis

6. Bilateral myelographic deformity

7. Favorable response to body jacket

8. Low-back pain > sciatica

9. Pseudarthrosis after previous fusion

B. Posterior Annular Tear Syndrom

1. abnormal discogram alone

2. Abnormal disogram: =/– CT and myelogram

TABLE 5 – FUSION AT ONE YEAR: 102/105 (97%) FUSED

SINGLE-LEVEL PLIF	INCIDENCE
L2-L3	2/2
L3-L4	6/6
L4-L5	59/61
L5-S1	28/29

TWO-LEVEL PLIF	INCIDENCE
L3-L4 & L4-L5	2/2
L4-L5 & L5-S1	4/4

TWO SINGLE-LEVEL PLIFS	INCIDENCE
L4-L5, L5-S1	1/1

TABLE 6 – SUMMARY:
ECONOMIC / FUNCTIONAL SCORES FOR 105 PATIENTS

RESULTS	SCORE	N	PERCENT OF TOTAL
Excellent	(9, 10)	45	
Good	(7, 8)	43	84
Fair	(5, 6)	14	
Poor	(2, 4)	3	16

TABLE 7 – SPONDYLOLISTHESIS (13/105)

CONGENITAL (5)	DEGENERATIVE (8)
L3-L4 (1)	
L4-L5 (1)	L4-L5 (6)
L5-S1 (3)	L5-S1 (2)

TABLE 8 – FAILED BACK (22/105)

POSTOPERATIVE ECONOMIC/FUNCTIONAL SCORE	NUMBER OF PATIENTS	
4 (Poor)	2	
5 (Fair)	0	
6 (Fair)	4	
7 (Good)	7	
8 (Good)	3	16/22 = 72%
9 (Excellent)	4	
10 (Excellent)	2	
Private Insurance	7	
Workman's Compensation	15	

TABLE 9 – COMPLICATIONS

Transient weakness, plantar flexor	(2)
Transient weakness, dorsiflexor	(1)
Transient bladder atony	(1)
Transient brachial plexopathy	(1)
Nonfatal pulmonary infarct	(1)

Absent: Death

Retropulsion of bone blocks

Infections

CERAMIC MATERIALS AND BONE REGENERATION

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ABSTRACT

Ceramic materials are being employed for the fabrication of many types of orthopedic and dental implants. Relatively inert metallic oxides are being used for the fabrication of components of total hip replacement prostheses (viz., femoral heads) and endosseous implants. These substances also occur as the passivation layer on all of the metallic materials currently employed for the fabrication of implants (e.g., chromium oxide and titanium oxide). Reactive ceramics including calcium phosphates are currently being investigated in particulate and bulk forms as bone substitute materials. Plasma sprayed hydroxyapatite (HA) coatings are serving as the attachment vehicle for joint replacement prostheses and dental implants. There has been recent interest in calcium phosphates as delivery systems for bone growth and inductive factors.

The clinical performance of bone substitute materials is related to the mechanical properties (including stiffness), porosity, and surface area. Synthetic HA implants are very dense and stiff and can, therefore, adversely influence the remodeling of surrounding bone. Moreover, their density and hardness prevents subsequent drilling of the implant site. In comparison anorganic bone has properties that more closely resemble those of natural bone. Moreover, the large surface area of anorganic bone commends its use as a delivery system for antimicrobials and agents such as growth factors.

Studies indicate that bone can bond to many types of calcium-containing ceramics. It appears that there is a common mechanism of bone-bonding related to the deposition of biological apatite onto the surface of the implant. The rate of bone formation on and around the surface of the substances may be related to the time period required for the deposition of biological apatite. In this regard substances such as natural bone mineral, that already comprise biological apatite, could be beneficial.

Synthetic calcium phosphate material does not undergo physiological remodelling, characterized by osteoclast-mediated resorption followed by bone formation. Evidence of osteoclast-like cells on the surface of anorganic bone suggests that natural bone mineral remodels in a more physiological manner.

INTRODUCTION

Because of its capability for regeneration (as opposed to repair with scar tissue) most wounds/defects in bone can be expected to heal without complications. However, excessive movement during the healing process can disrupt the stroma that serves as the framework for the regenerating bone, leading to the formation of fibrous tissue (scar). Many types of fractures require internal or external fixation to prevent this movement. Orthopedic implants such as total joint replacement prostheses need to have designs that provide mechanical stability during the healing process so as to allow the device to become osseointegrated. In these clinical situations gaps might exist between the bone ends or between the bone and implant. If the gap is sufficiently large (e.g., greater than a few millimeters) the bone regeneration process might not extend into the central portion of the gap, leading to the formation of fibrous tissue. Similarly, larger defects in bone, resulting from the excision of cyst or tumor, may not become completely filled by regenerating bone. Unfortunately, it is difficult to predict which gaps or defects will not undergo complete bone regeneration, because of differences in the available pools of pre-osteoblasts, vascularity, and other factors. One of these factors appears to relate to the stromal framework available to support cell migration and proliferation. Previous studies have shown that gaps will become completely bridged by regenerating bone trabeculae if filled with particles of allograft or a suitable bone substitute material. An additional benefit of these substances is to serve as a grouting agent to provide additional mechanical stability at the wound site during healing. Most substances being investigated as bone substitute materials are ceramics. Most of this paper is drawn from a recent review of this subject.[1]

Generally ceramics are combinations of metallic and non-metallic elements. Most often this term refers to metallic oxides, nitrides, or borides. However, this term can also be extended to include phosphates and silicates. In most cases ceramics are ionically bonded crystalline materials. However, noncrystalline glasses have also been included in this category.

Metallic oxides represent a large portion of the ceramic materials commonly used in the fabrication of orthopedic, dental, and maxillofacial implants. These include substances such as aluminum oxide (alumina), but also represent the type of surface layer that appears on the surface of all metals currently employed in medical application (e.g., chromium oxide on stainless steel and cobalt-chromium alloys, and titanium oxide on commercially pure titanium and titanium alloys). It is this ceramic film on metals that imparts corrosion resistance. While this class of ceramics is relatively inert other types of ceramics, particularly calcium phosphates, are exceedingly reactive when implanted in the body.

The more inert ceramic materials including aluminum oxide and zirconium oxide have been used in polycrystalline and single crystal forms for the fabrication of components for total joint replacement prostheses, particularly femoral heads. These ceramics materials have also been used for many years for the fabrication of endosseous dental implants. However, there have been relatively few applications in which these ceramics have been used in particulate or bulk form as bone substitute materials. It has generally been the calcium-containing phosphates, carbonates, and silica glasses that have been employed as bone substitute materials to fill defects in bone, augment fracture fixation, and to facilitate fixation of joint replacement prostheses. Because there is evidence that bone chemically bonds to these substances some have been used as coatings for prostheses (viz., plasma-sprayed hydroxyapatite coatings on femoral stems).

Disadvantages of ceramics for use as replacement for bone include brittleness, low fatigue strength as a result of notch sensitivity, high modulus of elasticity, and difficulty of fabrication. In addition, uncertainty remains about how their chemical composition and structure influence solubility and biodegradation/resorption.

The concept of using synthetic calcium compounds to augment or substitute for autogenous and allogeneic bone grafts dates back to the late 1800s when investigators implanted calcium sulfate in defects in bone.[2] In recent years there has been a dramatic increase in the types of synthetic calcium-containing substances undergoing investigation as bone substitute materials. This recent surge of interest is being driven by 1) concerns about the transmission of disease arising from the use of allografts, 2) findings that bone can "bond", and thus incorporate, a wide variety of synthetic calcium-containing substances, 3) interest in substances for the controlled release of drugs, growth factors

and other agents, and 4) technological advances in making bioceramic implants. However, despite the fact that a myriad of new bioceramics have been investigated as bone graft substitute materials, limitations in their performance compromise their utility. These limitations relate to the mechanical, physico-chemical (solubility), and biological properties. Difficulties in determing these properties, as well as the chemical composition and structure of the materials, have complicated the development of improved bone substitute materials. As more deficiencies in synthetic bone substitute materials come to light, a better appreciation is obtained for the unique characteristics of natural bone.

Generally, bone substitute materials have been developed to be either permanent (i.e., nonresorbable) or resorbable. However, questions have arisen regarding the rate at which all of these substances (even those referred to as permanent) undergo dissolution/degradation. Problems relate to uncertain rates of degradation, and the biological processes associated with this behavior. In some instances the implants have been found to undergo such rapid degradation that they provided little benefit as bone substitute material. Other questions relate to the biological processes mediating the resorption of these substances. For example, the phagocytic/endocytic processes associated with biodegradation could lead to non-specific resorption, and actually impair healing at the site.

Initially, bone substitute materials were developed to serve as a temporary scaffolds or "templates" to facilitate osteogenesis. Ultimately, the bone substitute material was to be "resorbed". Calcium sulfate[2] and tricalcium phosphate[3, 4] were two of the calcium-containing substances first studied. Potential problems with these materials were related to the fact that they often underwent physiochemical dissolution too soon after implantation for many applications.

Studies in the late 1960s and 1970s investigated tricalcium phosphate (TCP) as a bone graft substitute material.[3-5] Many of these early investigations focused on oral and maxillofacial applications. The particular substance employed was the beta form of TCP, produced by heat treatment processes. It also was referred to by its mineral name, whitlockite. This substance was generally used for applications in which a "resorbable" bone substitute material was required. Many of the studies conducted to date have focused on the time course of bone incorporation and degradation of this substance.[4,6-13]

However, there is still some question as to the rate at which this material becomes absorbed into the body. Differences in experimental findings might be related to variations in the composition and structure of the substance referred to as TCP, and the different physiological characteristics of the implant sites and animal models. Uncertainty about the bonding of bone to this substance is probably due to the fact that in many situations the TCP undergoes physicochemical dissolution at a rate which precludes the precipitation of biological apatite and subsequent bone formation on its surface; the dissolving surface does not allow for protein adsorption and cell attachment. Recently TCP was investigated as a carrier for a bone inducing substance, bone morphogenetic protein (BMP). An aggregate of BMP with TCP yielded a 12-fold greater amount of new bone than BMP alone in an animal (mouse) model.[14] "Whether TCP acts as a slow-release delivery system, potentiates the the activity of BMP, or serves to distribute BMP in a favorable three-dimensional pattern requires further investigation."[14] The search for more permanent "biocompatible" bone substitute materials generally focused on hydroxyapatite (HA), as it was considered to be the primary mineral constituent of natural bone. However, a few groups have been studying calcium-containing "bioactive glass ceramics".

Hench *et al.*[15-17] developed a partially soluble silicate glass containing calcium and phosphate. Partial dissolution of the surface in aqueous solution led to the formation of a gel-like substance with high concentrations of calcium and phosphate ions; this resulted in precipitation of HA on the surface. They referred to this class of calcium-containing reactive glassy materials as "bioactive glasses" and their particular formulation as Bioglass. In early animal studies they found that bone formed directly on the surface of Bioglass.[15] In later investigations of the biological response to bioactive glasses with a wide range of compositions in the $SiO-CaO-HPO_4$ phase system it was found that only certain formulations allowed for "direct bone bonding."[17-21] Despite the fact that is was the originally intended application, the limited strength of these glassy substances has prevented their use as coatings for joint replacement prostheses. Investigations are continuing to determine if there are ways in which these substances can be used as bone substitute materials. While these materials have not yet been shown to be of significant orthopedic value their investigation has provided insights into the bone bonding process that has assisted development of other calcium-containing substances.

Much of the early work with HA began in the mid 1970s. [12,22-24] Since that time many studies have focused on the use of hydroxyapatite, in dense and porous forms of block and particles, as bone substitute materials for orthopedic, dental, maxillofacial, and otological applications.[25-35] HA-like coatings flame sprayed onto metallic orthopedic and dental implants have also been undergoing intense investigation.[36-41]

With the increasing interest in investigation of bone substitute materials has come an advance in our understanding of the bone response to these substances. Mechanisms underlying the "bone bonding" associated with incorporation of these substances in bone are beginning to be revealed. However, despite these advances, limitations in the ability of synthetic bioceramics for duplicating the behavior of natural bone remain. These limitations relate to the fact that the synthetic products do not replicate the chemical composition and structure of natural bone mineral, which is a calcium-deficient carbonate apatite. This has prompted investigation of methods to treat natural bone products in order to prepare natural bone mineral to be used as an implant material.

PERFORMANCE REQUIREMENTS AND CHARACTERISTICS

Bone substitute materials are employed primarily to serve as fillers and scaffolds to facilitate bone regeneration. In order to be able to successfully function in this capacity the bone substitute should serve as a substrate for osteogenesis. In so doing it will become incorporated into host bone in such a way as to prevent migration, or movement relative to surrounding bone, that might interfere with the bone formation process. It is the bonding of bone to the surface of the implant material that facilitates its incorporation.

Bone substitutes are often employed to prevent collapse of surrounding bone into a defect. It is the strength of the bone substitute material that is important in determining whether it can adequately serve in this capacity. The modulus of elasticity of the implant material is important in its influence on the adaptive remodeling of surrounding bone. Substances that are very dense and stiff adversely influence the remodeling of surrounding bone, leading to nonanatomic remodeling with bone loss occurring in some areas and densification in others. The stiffening effect associated with implantation of high modulus ceramic materials can be particularly problematic in subchondral areas

where the overlying articular cartilage would be at risk for degradation because of the subchondral stiffening.

The resorbability of the bone substitute material can be advantageous, particularly for substances whose mechanical properties differ greatly from natural bone and whose long-term presence could jeopardize the function of surrounding tissue. However, is is important that the resorption process not occur so rapidly as to prevent incorporation.

Another potentially valuable function of bone substitute materials is as a carrier for the controlled release of antimicrobial, mitogenic (e.g., growth factor), and/or inductive agents. In this regard the surface area of the substance and its resorbability are of particular importance since they dictate the amount of the drug that can be adsorbed by and incorporated into the implant. Because of its large surface area due to the presence of cell lacunae, canaliculi, vascular channels, and Haversian systems, deorganified bone is commended for use as a drug delivery system.

In some applications the site of implantation might have to be drilled at a later time to accommodate fracture fixation devices or prostheses. Because of their very high density and hardness synthetic hydroxyapatite substances cannot be used for applications requiring drilling procedures. The lower hardness of bone mineral allows it to better accommodate drilling procedures.

In light of above, the characteristics that are important in relation to the performance of bone substitute materials include mechanical properties and the biological response (bone bonding and resorption). In order to better understand how bone substitute materials function, we need to be able to correlate these characteristics with the chemical composition and structure of the substances.

CHEMICAL COMPOSITION AND STRUCTURE

Determination of the chemical composition and crystalline structure of bone substitute materials is important 1) for research purposes in order to determine relationships between molecular structure and properties, 2) to verify the identity of bone substitute materials proposed for clinical implementation, and 3) for control of the quality of products being manufactured commercially. Chemical methods can be used to determine the concentration of calcium, phosphorus and other elements in a bone substitute material. Infrared spectroscopy (IR) can be useful in revealing the presence

of certain molecules and bonding. X-ray diffraction procedures reveal the crystalline structure of the substance. These methods are of particular value for analyzing calcium phosphate compounds because of the wide variation of the crystalline structure, and because of the cationic and anionic substitution that occur due to the highly isomorphous structure of apatite.

Infrared spectra for synthetic HA substances are different from those produced by natural bone and certain preparations of anorganic bovine bone (e.g., Bio-Oss, Geistlich-Pharma, Wolhusen, Switzerland). The IR spectra for bone displays the presence of carbonate and fewer hydroxyl ions than found in synthetic HA. The x-ray diffraction pattern of synthetic HA reveals the highly crystalline structure while poorly crystalline apatitic patterns are obtained from bone mineral. These findings demonstrate important differences between synthetic HA and natural bone mineral. The latter is a poorly crystalline carbonate-containing apatite. These chemical and crystalline differences between synthetic and natural bone mineral can result in differences in the biological performance of the substances.

BIOLOGICAL RESPONSE

Some of the earliest studies employing calcium phosphate ceramics in the mineral form of hydroxyapatite were reported by Jarcho.[22] His animal investigations yielded histology suggestive of bone bonding to the material. Jarcho interpreted the results of electron microscopy studies as showing the precipitation of bone apatite on the surface of implants.[22] It was postulated that collagen fibrils synthesized by nearby osteoblasts initially formed a zone between the calcified layer on the implant and the bone-forming cells. The continuum of hydroxyapatite-like crystallites was then generated between the calcium phosphate specimen and the matrix of the newly forming bone. Jarcho also suggested that there was evidence that the zone immediately adjacent to the implant material included glycosaminoglycans similar to those comprising the "ground" or "cementing" substance of natural bone. Later electron microscopy studies revealed other ultrastructural features of the interface between HA-like implants and tissue.[42-45]

Heughebaert *et al.*[46] employed methods to detail the composition and structure of synthetic HA implants before and after implantation in soft tissue sites in animals. They found that the mineral phase which deposited on the implants *in vivo* had characteristics consistent with bone apatite. The precipitating substance was found to be a

carbonate-apatite with an electron diffraction pattern and infrared spectrum similar to that obtained from bone mineral. This mineral phase was significantly different from the carbonate-free synthetic HA ceramic implant.

Recent investigators in France[47-53] and Japan[21, 54-58] have employed *in vitro* and *in vivo* models to evidence the deposition of biological apatite onto certain calcium phosphates and bioactive glasses in the course of bone bonding. One recent study proposed that the time required for deposition of apatite *in vitro* from a "simulated body fluid" could be correlated with the relative strength of bone bonding to various bioactive glass and HA substances.[58]

In vitro studies and animal investigations indicate that biological apatite deposits on the surface of calcium-containing implants soon after insertion into the body. This biological apatite layer presumably serves as a substrate for subsequent protein adsorption and bone cell attachment. The fact that biological apatite deposition is an obligatory precursor phase for bone bonding would indicate that implants comprising natural bone mineral might be more rapidly incorporated into host bone because their surface already comprises biological apatite. This is one of the rationales for employing anorganic bovine bone as a bone substitute material.

The terms "permanent" and "resorbable" have generally been used to describe the two broad classifications of bone substitute materials. In fact, all of the substances referred to as being permanent undergo some degree of biodegradation. This might take the form of physicochemical dissolution and/or fragmentation. The term resorbable has been used somewhat indiscriminately to describe the loss of implant substance over time. Strictly speaking, this term implies that the mechanism of breakdown of the synthetic material is the same as that occurring with osteoclast-mediated bone resorption. However, this is not been evidenced to be the case for synthetic calcium phosphate substances.

Results to date indicate that as that phosphate undergoes physicochemical dissolution and fragmentation, apparently at a rate related to the density of the material. The large differences in dissolution rates reported in animal investigations suggest that there are differences in the chemistry and structure of materials that have generally been termed tricalcium phosphate. The mechanism of breakdown of this substance *in vivo* is not fully understood. The appearance of macrophages and multinucleated foreign

body giant cells around this substance, when used in certain applications suggest that TCP particles might provoke the activation of phagocytes that in turn stimulate other cells and an inflammatory response. Agents produced by these cells could accelerate the degradation process.

While it is generally been considered a nonresorbable material, synthetic hydrox-yapatite has also been found to undergo physico-chemical dissolution, albeit at a very slow rate. Because it is only slightly soluble in biological fluid, synthetic HA substances can functionally be considered as long-lasting implants, especially when they are incorporated into bone.

In order to begin to determine the effects of chemistry and structure on the osseous response, synthetic HA (Calcitite, Calcitek, San Diego, CA) and natural bone apatite (Bio-Oss) were implanted in an animal model for bone regeneration.[59] Particles of each material, 0.5-1.0 millimeters were implanted into cylindrical defects prepared in the medial condyles of 30 rabbits. Animals were sacrificed 10, 20, and 40 days after implantation. After sacrifice the distal femurs of the animals were fixed in formalin and embedded in plastic for undecalcified "ground section" histology.

Histologically new bone formation was found on the surface of both types of implant materials ten days after implantation. The percentage of the surface of the particles covered by new bone and the percentage of the interstitial space filled with osseous tissue were comparable for sites implanted with the synthetic and natural substances. There was a trend suggesting that there was more bone within the defects filled with the natural bone mineral.

Analysis of the area fraction of implant material within the defects revealed a reduction in the amount of natural bone mineral suggesting absorption of this substance. Histology revealed the presence of greater numbers of osteoclast-like cells on the surface of the Bio-Oss[60] than found on the synthetic HA samples.

These results indicate that, in the time frame investigated, natural bone mineral and synthetic HA were incorporated at comparable rates. Other studies investigating shorter time periods of implantation may reveal differences in the rate of bone formation on the surface of the synthetic and natural bone mineral specimens. Unlike the synthetic material, it appeal. that the natural bone mineral crystallites of Bio-Oss undergo resorption by osteoclast-like cells.

CONCLUSIONS

Many interrelated factors influence the performance of bone substitute materials in facilitating the regeneration of osseous tissue. While the material might be selected primarily to serve as a delivery system for bone growth and inductive factors, its mechanical and solubility properties need to be considered also in terms of the functional requirements of the clinical application. Moreover, the biological responses associated with incorporation (i.e., bone bonding) and biodegradation need to be understood for the judicious use of the material. Synthetic ceramics differ from bone mineral in many important features of chemical composition, structure, and mechanical and biological properties. This serves as the rationale for use of anorganic bone as a delivery system for drugs and other agents.

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POLYMERS FOR BONE REGENERATION: REQUIREMENTS AND APPLICATIONS

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ABSTRACT

Polymers used for regeneration, rather than replacement, of lost bone must be biodegradable. They must degrade in concert with new bone growth, not interfering with, but rather promoting formation of new bone in osseous defects. They must also be convenient for the surgeon to use. The required end result is repair of the osseous defect with new bone that is structurally and physiologically equivalent to that which was lost. There are many possible ways to achieve these and several other known requisite properties in osteoregenerative devices. Here we review these requirements, citing examples and important current applications, highlighting work completed and in progress at the U. S. Army Institute of Dental Research (USAIDR).

INTRODUCTION

Ideal repair of osseous defects demands the regeneration of physically and physiologically competent new bone rather than permanent replacement of the lost bone with a foreign material. This is currently accomplished by grafting autogenous bone or implanting allogeneic bone. Both treatments have serious drawbacks. Autogenous material must be harvested from a donor site in a patient already compromised by disease, injury or age. Boyne and Zarem[1] and Lawson et al.[2] report that 9–19% of bone grafts to the craniofacial complex fail to produce osseous union, become necrotic, and are resorbed.[1,2] Autogenous grafts for segmental appendicular reconstruction have a reported failure rate of 50%.[3]

Allogeneic implants have an even more dismal record than autografts. There are several potential problems. First, these preparations are not standardized and do not yield predictable results. Also, allogeneic bank bone is immunogenic.[4] The possibility

351

of transmission of pathogens such as hepatitis B and the human immunodeficiency viruses are additional disadvantages. When used alone, bank bone is susceptible to resorption over time and it incorporates slowly with host bone, leading to fracture and infection in the recipient bed. Finally, delicate contours of the craniofacial complex and mandible are difficult to restore using either bank bone or autografts.

Osteoregeneration without the use of bone grafts is orders of magnitude more challenging than bone replacement. Development of synthetic polymeric matrices promises the ability to tailor physical and chemical properties of potential bone–regeneration materials to stringent physiologic specifications. Required chemical properties include biocompatibility and biodegradability. The implant must neither induce adverse local tissue reaction nor be systemically toxic or immunogenic. It must degrade at a rate and in a manner which does not hinder revascularization and ingrowth of new bone, while preventing collapse of the surrounding soft tissue into the bony defect. Porosity plays major role in controlling degradation and encouraging tissue ingrowth.

For cranial and maxillofacial applications, physical strength is less important than formability. The surgeon must be able to conveniently sculpt the material to fit these curved and angled contours. However, stress resistance and weight-bearing capacity can be critical for implants in the appendicular skeleton and mandible.

For military applications in particular, storage stability is another important requirement. Bone–regenerative materials must have extended shelf life, on the order of two to three years, without refrigeration or other special conditions. The goal is to make these materials deployable far forward on the battlefield so that surgical repair can be initiated as soon as possible after trauma. This capability will increase the likelihood of successful bone repair and help maintain troop strength by enabling soldiers to resume their normal duties sooner.

The goal of bone–repair research is to discover how to extend the body's ability to regenerate bone where critical amounts are missing due to developmental deficit, surgical removal, or traumatic avulsion. There is a mind–boggling list of requirements for materials with this capability. Biocompatibility, biodegradability, physical strength, and other parameters are inextricably interdependent. The following is an overview of these requirements and their interrelationships.

BIOCOMPATIBILITY

The term 'biocompatibility' encompasses both the reaction of the host to the presence of the implant and the effects of the host's physiologic environment on the implant. Considerations which might fall under this heading include: 1) local tissue reactions, 2) systemic toxicity, 3) allergenicity, and 4) cell anchorage.

In order for a synthetic implant to successfully regenerate tissue, the recipient must not react to it as though it is a foreign body. That is, the implant must not become encapsulated in fibrous tissue. Rather, it must become integrated into the host tissue defect, where the matrix will biodegrade as it is replaced by new tissue identical to that which was lost. Several processes must occur to support this.

First, limited acute inflammation due to trauma and surgical repair will spur angiogenesis and neovascularization. Establishment of local circulation is essential to support tissue ingrowth and remove degradation products from the implant site. Obviously, chronic inflammation due to irritation, sensitization, or allergy will result in implant failure. Sources of undesirable inflammation may be either physical or chemical. Surface geometries such as porosity, smoothness, or roughness may trigger vigorous cellular response. Surface hydrophobicity and charge are also critical. Polymer degradation products and residual solvents and sterilants must all be nonirritating and nontoxic.

When an implant is exposed to physiological exudate in a wound site, surface–active molecules such as plasma proteins are quickly and selectively adsorbed, modifying the polymer surface and paving the way for cell attachment. Clearly this process works better on some polymer surfaces than others, but it occurs most readily on inert, hydrophobic surfaces.[5]

Next, the physical characteristics of the implant surface must welcome attachment of osteoprogenitor cells. Not only are they anchorage dependent, but if they are analogous to other types of cells, preosteoblasts must also be supported in the proper shape and orientation in order to function as osteoblasts.[6,7] Sponge–like polymer matrices with high void volume similar to natural bone matrix are favored. These can be created from either natural or synthetic polymers. However, the latter permit more freedom to adjust surface chemistry, are more easily manipulated, and con be made nonimmunogenic.

Biocompatibility of bone–regenerative material must be accompanied by the capacity to induce formation of new bone in a site where it would not form otherwise. The term osteoinduction has been coined to describe this process of switching on the bone–producing machinery of the population of precursor cells which must find their way, or be seduced, into the host recipient bed.

OSTEOINDUCTIVITY

In order for skeletal defects to heal by osseous, rather than fibrous union, the combination of blood–vessel growth and proper cell attachment must be augmented by controlled delivery of osteoinductive proteins and growth factors. Bone has remarkable regenerative capacity, but when bc.ie loss exceeds a critical size, the normal cascade of events in the healing process must be supported by a properly scheduled release of those proteins which turn on bone expression in preosteoblasts. Essentially, the sequence of events which occurs in embryonic bone formation must be repeated in the defect site.[8]

Several osteoinductive proteins[9,10] and skeletal growth factors[11,12] have been isolated from bone using various purifications schemes. Some are known to have considerable amino acid sequence homology. These peptide sequences are highly conserved among animal species.[13] This has proven true for other proteins involved in critical wound–healing and tissue–repair mechanisms.[10] These proteins are believed to originate from a subclass of the transforming growth factor beta family. The have been cloned by several biotechnology firms, and will soon become available in much larger quantities for research applications.

Several growth factors involved in wound repair are known to be, or are suspected of being important in bone regeneration. They are believed to work in concert with osteoinductive proteins, amplifying the healing response. Work is in progress at USAIDR to evaluate the effects of selected growth factors in conjunction with the bone–inductive protein, osteogenin, in craniofacial defects.

Currently, much research in bone repair focuses on finding ways to incorporate osteoinductive proteins and growth factors into controlled–released delivery vehicles which also serve as scaffolds for tissue ingrowth. This is perhaps the most challenging aspect of development of bone–regenerative materials. Manipulation of most biode-

gradable, biocompatible polymers into porous matrices requires use of either organic solvents, heat, or both. Both are known to destroy the biological activity of many proteins. Osteoinductivity can only be conferred by predetermined release of needed amounts of active bone–inductive proteins and growth factors into the depleted osseous defect. Ultimately, this may best be accomplished by incorporation of cultured bone cells into implantable matrices, as is currently being done with cartilage and other tissues.[14,15]

OSTEOCONDUCTIVITY

The processes of blood–vessel ingrowth and diffusion of nutrients into and waste and degradation products out of an implant must occur within a porous matrix. Migration of bone precursor cells into the implant must not be hindered. Ideally, marrow space, which is the source of these cells, should not be occluded. The term osteoconduction describes the process of bone ingrowth from the margins of the wound bed. Osteoconduction should occur on the surfaces of permanent, nondegradabel bone implants as well, and is part of the process of osseointegration.[16] This insures physical strength of the bone–implant union.

Implant porosity and pore density must meet certain minimum specifications in order for osteoconduction to occur unimpeded. These specifications are still the subject of debate. A range of pore diameters has been reported to permit bone ingrowth. In ceramic matrices, a minimum pore diameter of 100 microns was required in one study[17], while another author recommends 500 microns as optimal.[18] Other studies focus on porous polymeric matrices with similar size, or larger pores.[19,20] High pore density (high void volume) maximizes diffusion and access to the interior of the implant and provides large amounts of surface area for cell attachment. However, there is a concomitant loss in compressive strength, which would be detrimental in stress–bearing sites. Optimal pore size and pore density will undoubtedly depend on the porosity and morphology of the original bone to be regenerated. Figures 1a and 1b are scanning–electron micrographs of natural bone matrix, clearly showing interconnected pores.

There are a variety of ways to construct porous polymeric matrices. Highly porous foams are desirable, and can often be made by lyophilization of a solvent from a polymer solution. Small particles of narrow size distribution, either microspheres of microfibers,

can be fused to form matrices with controlied, uniform pore size distributions. Figure 2 is a scanning-electron micrograph of such a matrix. Uniform pores can also be created by incorporating particles of a substance with different solubility properties than the polymer. This can be mixed into either polymer melts or solutions. The pore-forming compound is then dissolved out of the hardened polymer matrix, leaving interconnected pores (see Figures 3a and 3b). Several of these matrix architectures have been, and are being, evaluated in craniotomy defects at USAIDR.

BIODEGRADABILITY

If an implant is biocompatible, osteoinductive, and osteoconductive, it could be argued that it need not be biodegradable. However, whenever a foreign material is allowed to persist, complications almost inevitably develop. Permanent bone replacements and fixation devices often become loci for infection and must be removed. They may also loosen, resulting in weakness and fracture. No matter how well integrated a nondegradable bone replacement becomes, it will never be physically or physiologically equivalent to that which was lost.

A critical area in the formulation of bone-regenerative materials is the development of biodegradable, biocompatible polymers with predictable and controllable degradation rates. In addition to the familiar aliphatic polyesters, novel polyanhydrides[21], polycarbonates[22], and polyphosphazenes[23] have been synthesized. Novel polymers are also being produced by microbial fermentation (for example, GalactomedTM brand of polygalactosamine, Princeton Polymer Laboratories, Plainsboro, NJ).

Vascularity and the rate of bone regeneration in the recipient wound bed are factors which will help determine the selection of a particular polymer. Bone in the craniofacial complex is more richly vascularized than appendicular bone. Therefore, the same polymer implanted in both sites would be expected to degrade more rapidly in the craniofacial defect due to increased diffusion and mass transport within and and around the implant.

Biodegradation will result in the release not only of bone-inductive proteins and growth factors, but also of monomers, excipients, solvent, and perhaps sterilant residuals. All but the monomers can be minimized or eliminated. Preferably monomers

will be either completely inert or readily handled by the host's metabolic pathways. For example, some very interesting work is currently underway to synthesize novel polyamino acids using trifunctional amino acids, forming other than the natural amide linkages. These polymers degrade into the same amino–acid monomers as would naturally occurring polypeptides.[24]

Biodegradation can occur either by surface erosion or bulk degradation, or both. From the standpoint of controlled delivery of active proteins, as well as the requirement that an implant prevent collapse of soft tissue into the wound bed, surface erosion is probably the preferred mode of degradation. Bulk degradation could cause rapid release of large amounts of protein or other drugs as the implant crumbles into the defect.

A problem worthy of mention with respect to many hydrophilic biodegradable polymers is swelling which occurs as the polymer becomes hydrated. This could lead to poor fixation and contour restoration, and could be particularly dangerous in craniofacial sites near airways. Hydration prior to use is possible; however, long–term storage of biodegradable (usually hydrolyzable) polymers and proteins in aqueous buffers clearly is not an option.

While biodegradability is a desirable property of bone implants, it implies lack of stability under certain conditions. Storage stability should not be a critical problem under normal circumstances; however, at USAIDR the military medical mission requires bone–repair materials which can be stored and deployed as far forward on the battlefield as possible. In combat field hospitals, facilities will not be available to store materials under precise temperature control, or to support cells or tissues in culture. The difficulty probably will not be in stabilizing the polymer matrix, but in preventing deterioration of the biological activity of the osteoinductive proteins, growth factors, or cells carried within it.

STERILITY

Bone–regenerative implants for testing in animals and use in humans must be made sterile. When an implant reaches the stage of commercial production, it can be manufactured under sterile conditions. However, in the research and development phase, sterilization usually takes place after the implant has been constructed. Methods

such as exposure to ethylene oxide or cobalt–60 gamma irradiation have been used, but are known to cause unpredictable changes in the properties of the implant.[25] Sterilization by these methods can result in altered polymer degradation rates, introduction of deleterious chemical species, and destruction of the biological activity of any osteoinductive proteins carried in the implant.

PHYSICAL STRENGTH

Depending on the intended site of application, great physical strength is not always required of bone–regeneration materials. More important is the ability to simply prevent the surrounding soft tissue from collapsing into the defect and creating fibrous union, or misshapen bony contours. It is also important, particularly for craniofacial applications, that the surgeon be able to quickly trim the implant to fit using either a scalpel or rotary drill. Weight–bearing capacity would be more critical in materials intended for mandibular or appendicular reconstruction. However, regardless of the site, firm fixation is essential. For bone fixation, strength and stress resistance are critical properties. Poor fixation will cause even the optimal implant to fail.

OTHER REQUIRED BONE – REPAIR MATERIAL

The ideal bone–regenerative material will require positive fixation in order to insure osseous union. Degradable fixation devices exist and are being developed in the form of screws and plates. It should be possible to incorporate bone–inductive proteins into these devices.

Another highly desirable material is biodegradable bone wax for use as a hemostatic and as a mortar between bone stumps and implants. Bone wax is currently made from conditioned, purified beeswax. This material constitutes a foreign body whicn can break up into small, soft pieces, resulting in embolus formation.[26] Several proprietary formulations based on biodegradable synthetic polymers are under investigation in our laboratory. Certain bioadhesives have potential in this application as well. Degradable hemostatics could also function as carriers for osteoinductive proteins in certain applications.

OUTLOOK

The future of bone regeneration rests on three important areas of research. One is the development of novel, biocompatible, biodegradable polymers and new ways to manipulate those already available. These efforts combine the talents of synthetic organic chemists, polymer chemists, materials scientists, and bioengineers to develop synthetic bone matrices with controllable, predictable properties.

Another focus is on development of technology to make these matrices osteoinductive. This research draws on polymer chemistry, biochemistry, immunology, and cell biology. Methods are being developed to incorporate, stabilize, and release biologically active substances into depleted bone defects. These implants must perform as sophisticated drug delivery systems, as well as scaffolds for proper contour restoration.

The third crucial area of research involves plastic and reconstructive, maxillofacial, and orthopedic surgeons in the refinement of techniques for osseous wound repair and implantation and the development of reliable animal models. After all, surgeons will be the end users of bone–regenerative materials. Their patients will be the beneficiaries of this interdisciplinary technology. The ultimate goal is development of an off–the–shelf, artificial bone that will be used by surgeons to restore form and function without the disadvantages of autografts and bank–bone alloimplants.

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FIGURE   LEGEND

Figure 1 a and b

Scanning – electron micrographs of freeze-dried human cancellous bone matrix

Figure 2

Scanning – electon micrograph of a uniformly porous matrix of fused poly (lactide-co-glycolide) microspheres

Figure 3 a and b

Scanning - electron micrographs of experimental, porous, degradable polymer matrix (Princeton Polymer Laboratories, Plainsboro, NJ)
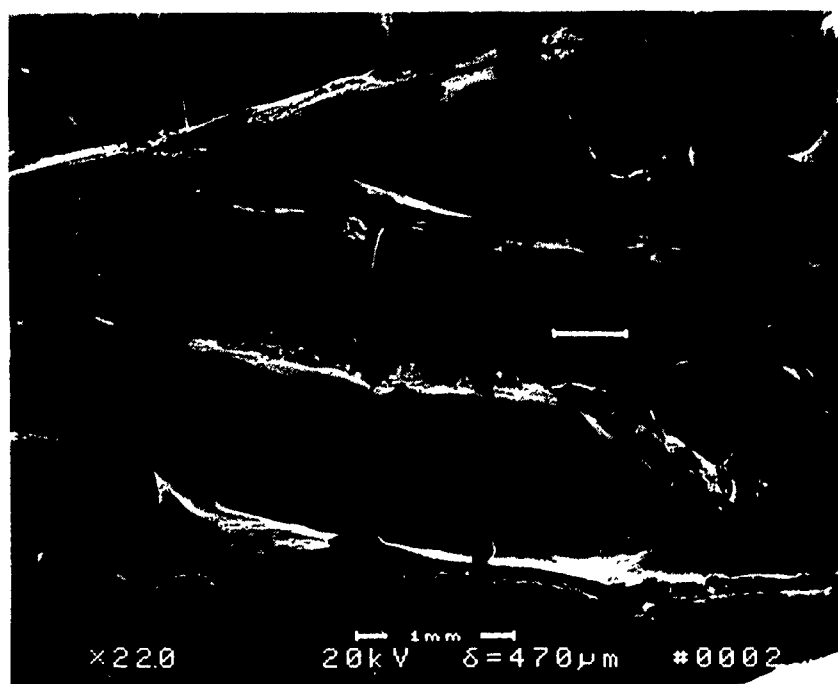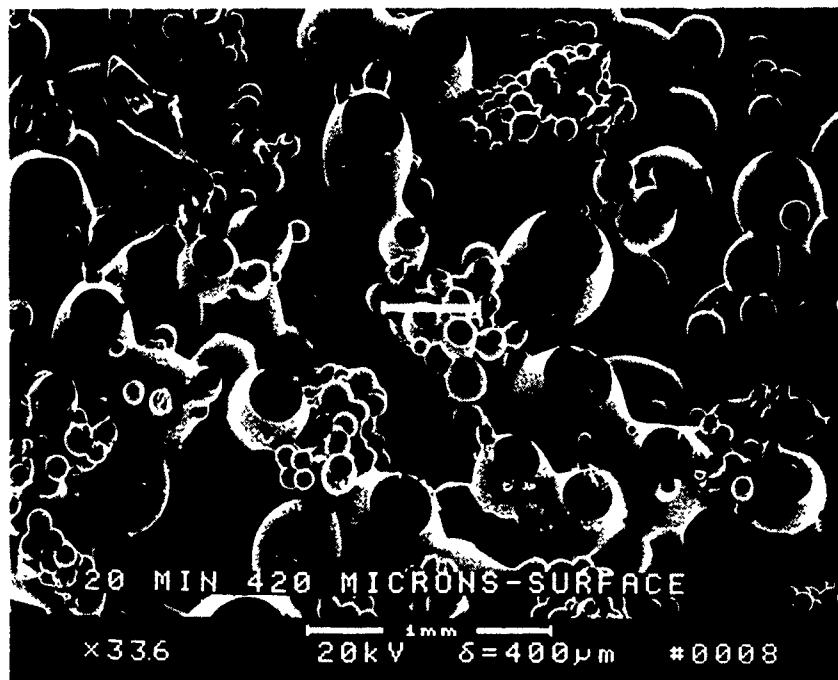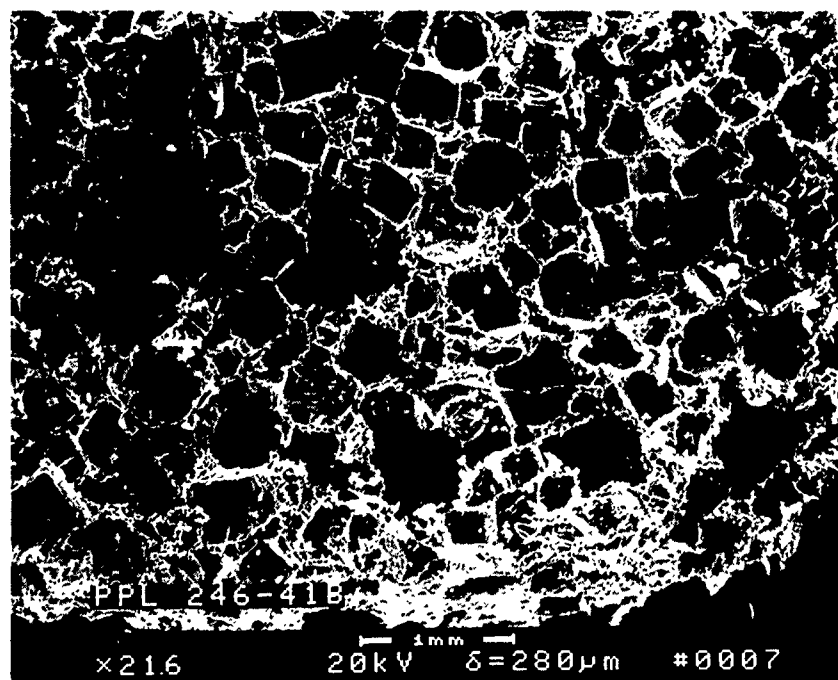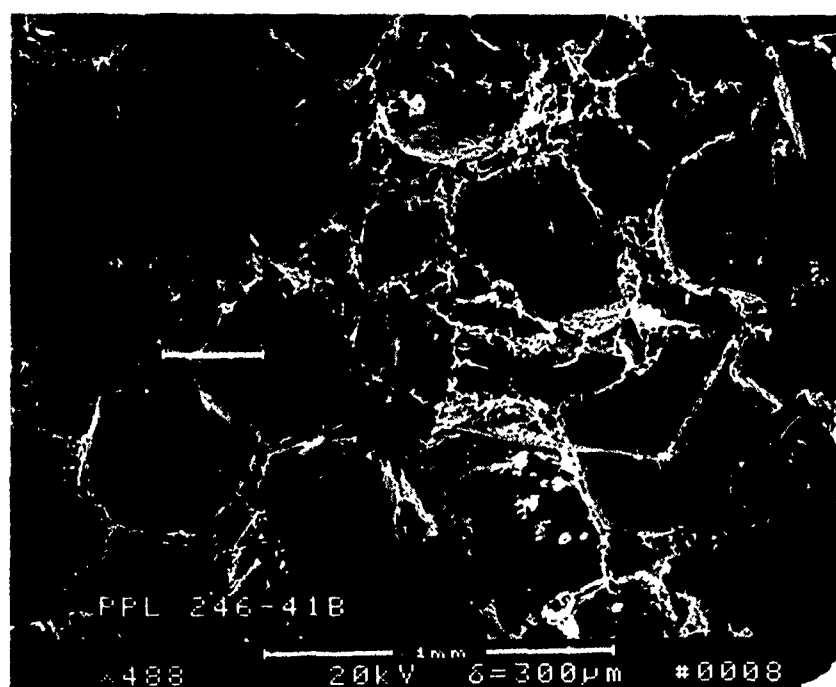
Figure 1a

Figure 1b

Figure 2

Figure 3a

Figure 3b

POLYMERS

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INTRODUCTION

Over the past decade there has been increasing attention devoted to the development of controlled release systems for drugs, pesticides, nutrients, agricultural products and fragrances. However, nearly all of the systems that have been developed have not been capable of slowly releasing drugs of large molecular weight (MW>600). In fact, up until 1976 it was a fairly common conception in the field of controlled release that effective systems could not be developed for macromolecules.[1] However, after several years of effort we discovered an approach that permitted the continuous release of biologically active macromolecules as large as 2,000,000 daltons from normally impermeable, yet biocompatible, polymers for over 100 days.[2] In this paper we review three areas of our research 1) systems that release large molecules through porous polymer matrices 2) novel biodegradable polymeric delivery systems 3) modulated controlled release polymer systems.

POROUS DELIVERY SYSTEMS FOR THE RELEASE OF
PROTEINS AND MACROMOLECULES

Prior to our studies, protein release could only be achieved very rapidly because these large molecules would only diffuse through highly porous and permeable membranes such as millipore filters or gels such as polyacrylamide. We then developed the first approach that permitted sustained release of large molecules from biocompatible polymers.[1] We dissolved the polymer in an appropriate solvent and added the macromolecule in powder form. The resulting mixture can be cast in a mold and dried. When the pellets are placed in water, they release the molecules trapped within the polymer matrix.

We tested a number of polymer systems for tissue biocompatibility and release kinetics. Our best long-term release results were obtained with hydrophobic polymers.

367

Examples included ethylene-vinyl acetate or polylactic acid. Certain hydrogels like polyhydroxyethylmethacrylate or polyvinylachol also worked effectively, but released the molecules trapped wthin the polymer matrix.

We tested a number of polymer systems for tissue biocompatibility and release kinetics. Our best long-term release results were obtained with hydrophobic polymers. Examples included ethylene-vinyle acetate or polylactic acid. Certain hydrogels like polyhydroxyethylmethacrylate or polyvinylalcohol also worked effectively, but released proteins for shorter time periods. With the hydrophobic polymers, biologically active protein was released for over 100 days. In other tests larger molecules (2 million mol wt), such as polysaccharides and polynucleotides, were also successfully released for long time periods.[1]

While these initial studies demonstrated the feasibility of releasing macromolecules from biocompatible polymers the kinetics were often not reproducible; we had not achieved controlled release. The irreproducibility resulted from drug settling and redistribution during casting and drying, caused by the insolubility of the incorporated macromolecule powder in the polymer solvent. At room temperature, the drug migrated vertically and visible lateral motion was caused by currents (possibly thermal) in the mixture. We thus developed a low-temperature casting and drying procedure to minimize this drug movement during matrix formation. By casting the dissolved polymer-solid drug powder mixture in a mold at -80 °C, the entire matrix froze before any settling could occur. These matrices were then dried at -20 °C for 2 days until almost all the solvent was gone. Final drying was conducted under vacuum at room temperature.[2]

With this reproducible method, we could now accurately assess factors that regulated release kinetics. We found that drug powder particle size and drug loading (drug:polymer ratio) influence release time.[6] We coated drug-containing polymeric matrices by dropping each matrix into polymer solutions of differing concentrations. The coated matrices were dried and tested for release kinetics. We found that an increase in coating solution concentrations significantly decreases release kinetics. By combining these simple fabrication parameters—drug particle size, loading, and coating—release rates for any drug could be changed several thousandfold.[2]

In vitro and *in vivo* release kinetics were compared using two different approaches. In the first approach (the recovery approach) polymer implants containing a radioactively

labelled substrate - ^{14}C-Bovine Serum Albumin, ^{14}C-β-lactoglobulin, or ^{3}H-inulin — were implanted subcutaneously into rats *(in vivo)* or released in phosphate buffered saline, pH 7.4, at 37 °C *(in vitro)*. At various time points, the polymer implants were removed from the rats or the saline and analyzed. Release rates determined in this manner were essentially identical *in vivo* and *in vitro*.[3]

One limitation of the above approach, however, was that we could not assay the amount of macromolecules directly released *in vivo*. This is because macromolecules such as proteins are metabolized, making direct *in vivo* release measurements difficult. To solve this problem we used ^{3}H-inulin as a model, which is totally excreted and is neither metabolized *in vivo* nor reabsorbed or secreted by kidney tubules. Thus, all inulin released from the polymer should be recovered in the urine. An *in vivo-in vitro* comparison ws made by making 9 identical inulin-polymer pellets. *In vivo* and *in vitro* release rates agreed to within 1% .[3]

Studies have also been conducted to explore numerous applications of these systems. These include release of insulin[4], anticalcification agents, interferons, growth factors[5] and inhibitors, and neurologically active agents.

BIODEGRADABLE POLYMERS

Biodegradable controlled release systems have an advantage over other systems in obviating the need to surgically remove the drug depleted device. In many cases, however, the release is augmented by diffusion through the matrix, rendering the process difficult to control - particularly if the matrix is hydrophilic and thereby absorbs water, promoting degradation in the interior of the matrix. To maximize control over the release process, it is desirable to have a polymeric system which degrades only from the surface and deters the permeation of the drug molecules. Achieving such a heterogeneous degradation requires the rate of hydrolytic degradation on the surface to be much faster than the rate of water penetration into the bulk. With this in mind, we proposed that an ideal polymer would have a hydrophobic backbone, but with a water labile linkage. Many classes of polymers, including polyesters, polyamides, polyurethanes, polyorthoesters, and polyacrylonitriles had been studied for controlled delivery applications, but only polyorthoesters erode from surface and then only if additives were included in the matrix. None of these polymers have yet received FDA

approval for human implantation. In designing a biodegradable system that would erode in a controlled heterogeneous manner without requiring any additives, we have suggested that due to the high lability of the anhydride linkage, that polyanhydrides may be a promising candidate. We have also developed several approaches for synthesizing these polymers. The most hydrophobic polymers displayed constant erosion kinetics over eight months. By extrapolation, 1 mm thick discs of polyanhydride will completely degrade in over 3 years. By altering the ratio of monomers in copolymers nearly any degradation rate between 1 day and 3 years can be achieved.[6]

We also conducted initial biocompatibility studies. As evaluated by mutation assays, the degradation aproducts of the polymer wer nono-mutagenic and non-cytotoxic. Teratogenicity test were also negative. Growth of mammalian cells in tissue culture was also not affected by these polymers.[7]

In 1985, we began a collaboration with a neurosurgery group headed by Dr. Henry Brem at Johns Hopkins to explore the possibility of implanting polyanhydride discs containing the nitrosoureas, BCNU and CCNU, for brain cancer following surgery. Surface erosion would be critical for such drugs for if bulk erosion occurred uncontrolled amounts of this potentially toxic drug could be released during breakup of the matrix. In 1987, the FDA approved these polyanhydrides for human clinical trials. Clinical trials have shown this to be a safe procedure and prolongs life. Other applications of these polymers include the controlled release of gentamycin for possible treatment of osteomyelitis.

MODULATED RELEASE SYSTEMS

Several polymeric systems capable of delivering drugs at increased rates on demand were studied and developed. The first system consists of drug powder dispersed within a polymeric matrix (generally ethylene vinyl acetate copolymer, EVAc), together with magnetic beads.[8] Release rates were controlled by an oscillating external magnetic field, which is generated by a device that rotates permanent magnets beneath the vials. By placing small plastic cages containing animals on the top disc, it can also be used for *in vivo* studies. Polymer matrices containing drug and magnets can release up to 30 times more drug when exposed to the magnetic field, and release rates return to normal when the magnetic field is discontinued. The magnetically controlled

implant does not cause inflammation *in vivo* . This was confirmed by the lack of edema, cellular infiltrate or neovascularization as judged by gross and histologic examination in animals. Using insulin as a marker the above effects observed in vitro (response time, response duration, amplitude) were also observed *in vivo*.

We also discovered that ultrasound could affect the release of substances from polymers. The ultrasound system has a potential advantage over many other systems in that no additional substance (e.g., magnetic bead) is required in the polymeric matrix. Furthermore, in the case of ultrasound the polymer can be injected, and since it can be erodible there is no need for surgical removal. The application of ultrasound in humans, both for diagnostic and therapeutic purposes, has been extensively studied and is considered a safe practice. Enhanced (up to 20 times baseline) polymer erosion and drug release were observed when bioerodible samples were exposed to ultrasound. The system's response to the ultrasonic triggering was also rapid (within 2 min.) and reversible. This was determined by an on-line UV spectrophotometric response in a closed loop detection system where the concentration of the releasing agent was continuously monitored.[9]

The enhanced release was also observed in non-erodible systems exposed to ultrasound where the release is diffusion dependent. This was tested on EVAc copolymers loaded with BSA or insulin. The released insulin was also evaluated by HPLC. No significant difference was detected between insulin samples exposed to ultrasound and unexposed samples, suggesting that the ultrasound is not degrading the releasing molecules.[9]

Experiments were also performed to evaluate if the extent of enhancement could be regulated externally. By varying the intensity of the ultrasound, the degree of enhancement for both polymer degradation and drug release for the bioerodible and non-erodible systems could be altered tenfold.[9]

In vivo experiments were performed using para aminohippuric acid (PAH) as a marker inside the polymers. Data of rat's PAH concentration in the urine after implantation shows that the PAH concentration in the urine is pronounced during the exposure and mainly in the timespan just after the exposure.[9]

We have finally developed an approach for feedback control of polypeptides incorporated within polymeric drug delivery systems. This approach is based on the

observation that changes in pH can cause dramatic shifts in the solubility of polypeptide drugs; solubility is one of the prime determinants of release rate in any diffusion, dissolution or osmotic controlled release system. The system components involve an external trigger molecule and a polymer-bound enzyme that, in the presence of the trigger molecule, will cause acid or base to form. To test this concept, we used insulin as a drug and diabetic rats as the animal model. We chose to adapt the ethylene-vinyl acetate (EVAc) polymeric insulin delivery system capable of treating diabetic rats for over 100 days. To establish feedback we utilized the fact that insulin solubility is pH dependent and that, in the presence of glucose oxidase, glucose is converted to gluconic acid. Thus, when this enzyme is incorporated within a controlled release polymer matrix, external glucose should theoretically reduce the pH in the polymer microenvironment. Since the isoelectric point of insulin is 5.3, when the polymer is exposed to the physiological pH of 7.4 a decrease in insulin solubility and release rate is expected. This undesired effect is overcome by using a modified insulin which contains more basic groups and thus has a higher isoelectric point. Tri-lysyl insulin with an iso-electric point of 7.4 was synthesized for this purpose. The feasibility of this enzyme mediated feedback mechanism was established by three sets of experiments: 1) the effect of glucose on the pH in the microenvironment of the polymer, 2) the effect of glucose on insulin release *in vitro*, and 3) the effect of glucose on insulin release *in vivo*.[10]

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COLLAGEN AND BONE REPAIR

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ABSTRACT

Implants fabricated from mixtures of solubilized bone proteins and collagen have been shown to be effective in enhancing the restoration of osseous defects in experimental animals. The physico-chemical properties of these implants render them ideally suited for restorations in the craniofacial areas. Implants of a similar nature could also be used in other portions of the skeletal system provided measures were taken to stabilize the region of restoration. To date, the efficacy of these implants when used in a spectrum of human patients has not been evaluated.

INTRODUCTION

There is a significant need to enhance the restoration of bone in several medical and dental specialties. The need arises from the requirement to recontour or replace osseous defects created by developmental anomalies, disease processes, trauma or surgical procedures. Current treatment modalities for osseous reconstruction include fresh autogenous bone grafts, allogenic freeze-dried bone grafts, demineralized bone powder, and augmentation by application of ceramics such as hydroxylapatite or tricalcium phosphate. Each of these approaches, whether osteoinductive, osteoconductive or both, may be effective in restoring osseous defects. However, there are a sufficient number of disadvantages associated with each modality to warrant the search for more efficient, reliable and manageable alternatives.

Approximately five years ago, we initiated a series of studies to evaluate the possibility of routinely and effectively utilizing the osteoinductive capacity of demineralized bone[1] in a clinical setting. It was felt that the most appropriate device for this purpose would take the form of an implant composed largely of a solid, yet

malleable, nonimmunogenic and biodegradable material which could serve as the carrier for the factor(s) requisite for stimulating new bone formation. This has led to the development of an implant system fabricated by combining collagen with bone-inductive factor(s).

METHODS

In anticipation that the implants might eventually be used in human subjects, our experiments have been performed utilizing highly purified human type I collagen. The latter is obtained following limited pepsin digestion of full-term placentae and selective salt precipitation of type I collagen from acidic and neutral solutions.[2] All preparations of collagen are evaluated for purity and the potential presence of other fiber-forming collagens by a recently described technique in which cyanogen bromide peptides derived from each batch are evaluated for the presence of marker peptides for the various fiber-forming collagens.[3] The carrier, then, is not simply insoluble bone particles which are at times mistakenly denoted as collagen by other authors. In initial experiments designed to test the feasibility of the implant system,[4] bone proteins were extracted from demineralized bone in solutions of guanidine-HCl[5] and further purified by anion-exchange chromatography[6] to enrich the crude extracts in bone morphogenetic factors. In subsequent experiments,[7] bone proteins were prepared as the water-soluble fraction of guanidine-HCl extracted material.[8]

In all cases, implants have been prepared by adding bone-derived proteins to solutions of collagen in 0.5 M acetic acid to achieve maximum dispersion of all components. The resulting solutions which contain 60% collagen and 40% bone-derived protein are then lyophilized and the dried proteins compressed to form circular disc-like implants or rectangular implants with a thickness 4.0 - 5.0 mm.

RESULTS

The collagen-based implants described above have several characteristics which would make them ideal for routine use in a clinical setting. Among these are: 1) physical and chemical stability allowing storage and use for various intervals; 2) malleability allowing both deformation and swelling as well as cutting and carving to fit the contours of virtually any defect; 3) limited or no

antigenicity; 4) flexibility allowing the fabrication of implants of various sizes and with graded amounts of bone-inductive factors; and most importantly 5) excellent performance records in promoting osteogenesis at ectopic sites[4] as well as at osseous (cranial) sites[7] when tested in experimental animals.

DISCUSSION

Although the implants described above appear to be well suited for clinical use, several questions still remain. It is not known, for instance, for how long and under what conditions such implants can be retained in reserve and still maintain their effectiveness. These variables should be evaluated since one of the chief advantages of a system of this nature is the potential to stockpile material prior to use. An additional major question concerns potential adverse reactions, particularly antigenic reactions to bovine bone proteins, when the implants are used in humans. This problem may not arise, however, if our experience in the animal systems can be directly applied to humans. If this problem appears when implants are used in humans, it may likely be circumvented by using more highly purified factor(s) which would lower the proportion of bovine proteins in the devices. Of course, the most crucial question remans: can these implants achieve in human subjects the same degree of osteogenic enhancement as we have seen in animal experiments? We will attempt to discern this possibility in a series of studies whereby the implants are used to promote cranial bone restoration in patients undergoing neurosurgical procedures.

In closing, one practical consideration is warranted. Namely, the implants described above are relatively easy and convenient to prepare from materials that are readily available. Our experiments on bone formation at ectopic sites[4] were conducted using implants made from human type I collagen carrier and rat bone proteins. However, the experiments on restoration of cranial bone[7] were performed with implants composed of human type I collagen carrier and bovine bone proteins. Thus, it would appear that both the carrier protein as well as the bone proteins may be derived from xenogeneic sources without compromising the efficacy of the implants. This is particularly important with respect to the bone proteins which would be extremely difficult to prepare in suitable quantities were it necessary to use even allogeneic materials.

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INSULIN-LIKE GROWTH FACTOR-II IS A POTENTIAL LOCAL REGULATOR OF HUMAN BONE FORMATION

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ABSTRACT

Human bone cells *in vitro* produce insulin-like growth factor-II (IGF-II), IGF binding proteins (IGFBPs) and express IGF-II cell surface receptors. IGF-II is the most abundant growth factor found in extract of human bone and has been demonstrated to stimulate bone cell proliferation and type I collagen synthesis, two necessary components of bone formation. Based on these findings, we propose that the IGF-II regulatory system (IGF-II, IGFBPs and IGF-II receptors) plays a key role in the local regulation of bone formation, including that of the coupling of bone formation to bone resorption.

INTRODUCTION

Bone has two major functions, mechanical support and an important metabolic role as a mobilizable source of minerals, especially calcium. Both of these functions are intimately related to bone volume which is maintained through a continuous balance, i.e., a coupling, between bone formation and bone resorption. This coupling is thought to be mediated by local growth factors. Growth factors in bone have been suggested to act as paracrine/autocrine effectors of bone formation and have been demonstrated to increase bone forming cell (osteoblast) proliferation and bone matrix biosynthetic activity.[1-5] A number of growth factors are produced by bone cells and deposited in bone matrix. This review is focused on one such growth factor, insulin-like growth factor-II (IGF-II) which is a key bone cell mitogen thought to be involved in the coupling of bone formation to bone resorption.

GROWTH FACTORS AND LOCAL REGULATION

In vivo, an increase in bone resorption is usually followed by an increase in bone formation, i.e., a coupling of bone formation to bone resorption.[6] Studies of this coupled change in bone formation showed that the increase in bone formation was 1) proportional to the amount of bone resorbed, hence the use of the term coupled, 2) site specific and 3) mediated, at least in part, by an increase in local osteoblast number.[7-9] Since this phenomenon was observed to be a local response, the direct action of systemic agents could be ruled out.[9] Thus, the hypothesis was advanced that this coupling phenomenon was regulated at the local level. *In vitro* studies showed evidence that a large molecular weight mitogen was released in cultures stimulated to resorb.[10,11] This factor stimulated bone cell proliferation *in vitro*[11] and in nondissociative extracts from the bones of a number of species including chicken,[12,13] human,[14,15] bovine[16-18] and rat[19] was present in high molecular weight forms. Dissociative extracts showed that the factor was complexed, presumably to a binding protein.[20,21] The factor was given the term, skeletal growth factor (SGF).

SGF was subsequently purified to homogeneity and structural studies of the amino acid sequences from the amino terminal region and several tryptic fragments of human derived SGF were shown to be identical to the corresponding human serum IGF-II sequences.[22] Approximately 70% of the sequence of SGF was obtained from these studies and showed identical structural sequence to IGF-II. In addition, a number of bioassays demonstrated identical responses for human bone derived SGF and human serum derived IGF-II.[22] From this data, it was concluded that SGF was extremely similar, if not identical to IGF-II. During the course of these studies on the purification of IGF-II, it was found that extracts of human bone contained mitogenic activity that was not attributable to IGF-II alone.[23] Subsequently, studies from several laboratories have shown that a number of growth factors are present in bone matrix extractions including IGF-I, transforming growth factor-ß1 (TGF-ß1), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). In addition, a number of bioactive factors (e.g., bone morphogenic proteins and chemotactic factors) have also been identified in dissociative extracts of bovine and rat bones.[24-27] These recent studies have provided evidence that several of the above mentioned growth factors may act as potential determinants of local bone formation. Thus, the coupling phenomenon probably involves all bone growth factors including IGF-II.

EVIDENCE THAT IGF-II IS A POTENTIAL LOCAL REGULATOR OF HUMAN BONE FORMATION

A. IGF-II is the Most Abundant Growth Factor Present in Human Bone

While there are a number of growth factors present in extracts of human bone matrix, IGF-II and TGF-ß are the most abundant. IGF-II is approximately three-fold in abundance over TGF- and fifteen-fold over IGF-I. There are relatively minor amounts of PDGF and bFGF present in human bone matrix.[28]

B. IGF-II is Produced by Bone Cells

A number of growth factors have been shown to be produced by human bone cells *in vitro*, including IGF-I, IGF-II, TGF-ß1, PDGF and possibly bFGF. Of these growth factors, IGF-II is the most abundant one produced by human bone cells.[28] Bone cells derived from a number of other species including chicken, mouse and rat have also been shown to produce IGF-II.[22, 29-33] However, in the bone cells of the mouse and rat, IGF-II production is substantially less than that of IGF-I.

IGF-II synthesis by bone cells is both systemically and locally regulated. Regarding systemic regulation, 1,25 vitamin D, which stimulates bone resorption, increased production of IGF-II in serum free cultures of newborn mouse calvaria.[34] In addition, the steroid hormone, 17-beta-estradiol, stimulated production of IGF-II in a rat osteosarcoma cell line, UMR 106.[29] Regarding local regulation, low amplitude, frequency specific electric fields, which have been hypothesized to mediate the local effects of exercise, increased the release of IGF-II in a mouse osteoblastic cell line and in human bone cells.[35] Thus, agents which modulate bone formation *in vivo* have been shown to affect IGF-II synthesis by bone cells *in vitro*.

C. IGF-II has Significant Biological Actions on Bone Cells

IGF-II has been demonstrated to be mitogenic for untransformed human femoral bone cells and to significantly increase the incorporation of [³H] thymidine into DNA, in a dose dependent manner.[36] In addition to the stimulatory effects on [³H] thymidine incorporation, IGF-II stimulates the synthesis of type I collagen in untransformed human bone cells and causes a rapid induction of a protooncogene, c-fos, in murine osteoblast-like cells.[37,38] These findings suggest that IGF-II plays a role in bone formation.

D. IGF-II and PTH Induced Uncoupling

A study of elderly patients with hip fractures revealed that these patients demonstrated an increased serum parathyroid hormone (PTH) level, probably due to calcium malabsorption.[39] In a calcium deficient state, there are two means by which an increase in the amount of calcium retrieved from bone can be accomplished: 1) increased bone resorption and 2) decreased bone formation. It is well understood that PTH can increase bone resorption. In addition, under calcium deficiency states, PTH is also associated with a decrease in bone formation which may in part be mediated by the IGF-II regulatory system. In this regard, it has been shown that PTH increased the production of a newly discovered IGF binding protein (IGFBP-4) which is a potent inhibitor of IGF actions. Furthermore, serum IGFBP-4 level was significantly elevated in serum collected from elderly, hip fracture patients.[39] Based on these studies, it is speculated that the IGF-II system may be involved in the PTH induced uncoupling of bone formation. Two findings support this concept: 1) IGFBP-4 inhibited basal bone cell proliferation by approximately 40% in serum free conditions suggesting that endogenous production of IGFs contribute substantially to basal bone cell proliferation[40] and 2) PTH stimulated the production of IGFBP-4 by human bone cells in vitro.[41]

PROPOSED MODELS FOR IGF-II ACTIONS

The findings that: 1) human bone cells produce IGF-II, 2) IGF-II stimulates proliferation and collagen synthesis in human bone cells and 3) IGF-II is the most abundant growth factor present in human bone matrix, implicates IGF-II as an important regulator of human bone cell metabolism and function. We propose the following model for the actions of IGF-II in the bone cell microenvironment (Fig. 1). Bone cells secrete IGF-II which is either directly incorporated into the bone matrix for future actions or diffuses into the local extracellular matrix. IGF-II secreted into the extracellular matrix can have acute effects on nearby bone cells (paracrine actions) or may affect the same osteoblast cells that produced it (autocrine actions).

Some of our most recent work supports the concept that IGF-II is fixed into the bone matrix by means of an IGFBP (bone derived IGFBP, i.e., BD-IGFBP) that displays strong affinity for hydroxyapatite and selective affinity for IGF-II over IGF-I.[42] IGF-II itself does not bind to hydroxyapatite. We have proposed a model (Fig. 2) in which IGF-II is

fixed into the bone matrix (by BD-IGFBP) where it remains in an inactive, stable form. Subsequent osteoclastic resorption of an area of bone matrix would release the once fixed IGF-II in a bioactive form. Once released, IGF-II could either stimulate precursor osteoblasts as to increase the number of mature osteoblasts or to stimulate already present mature osteoblasts to increase matrix biosynthesis or both. This would allow for the replacement of bone lost to resorption (i.e., couple bone formation to bone resorption) by means of a delayed paracrine mechanism.

REGULATION POINTS OF IGF-II ACTIONS

If IGF-II is an important local regulator of bone cell proliferation and differentiation, then one would anticipate that the actions of IGF-II be uniquely regulated in bone. In this regard, recent studies suggest that there are a number of potential control points in the IGF-II system of bone: IGF-II synthesis, IGF-II receptors and IGF-II binding proteins, which are described below.

IGF-II is first synthesized as prepropolypeptide with a relative molecular mass of 20 kd that includes a 24 amino acid signal peptide and an 89 amino acid carboxyterminal peptide.[43] Recent evidence suggest that IGF-II synthesis is regulated at multiple control points including transcription, differential polyadenylation, post transcriptional processing and precursor processing.[44] Northern analyses of IGF-II mRNA transcripts using a coding sequence cDNA probe revealed that human bone cells express multiple IGF-II transcripts, some of which are probably unique to bone.[45,46] However, which of these IGF-II transcripts is regulated or translated in bone cells remains to be determined. IGF-II is produced by a variety of cell types but the production is regulated differently depending upon the tissue type. It is proposed that the complexity of IGF-II gene regulation may allow for the tissue specific expression of IGF-II in bone.

Another potential point for the regulation of IGF-II could be through the modulation of IGF-II receptor number and/or affinity. In this regard, although the involvement of IGF-II receptors in mediating the proliferative effects of IGF-II is controversial, studies in our laboratory are consistent with the idea that the IGF-II receptor mediates at least part of the mitogenic actions of IGF-II in bone cells.[47] Furthermore, recent studies demonstrate that dihydroxytestosterone treatment enhances the mitogenic effect of IGF-II in bone cells by a mechanism that may involve increased IGF-II receptor

number.[48] In addition, insulin treatment increases IGF-II receptor number in MG63 human osteosarcoma cells, SaOS human osteosarcoma cells and in primary cell cultures of embryonic chick calvarial cells without affecting receptor affinity for IGF-II.[49] IGF-II receptors have been shown to be increased in parallel with IGF-II expression during myogenic differentiation and the distribution of IGF-II receptors in embryonic rat tissues, *in vivo*, parallels the distribution of IGF-II transcripts.[50,51] From these studies, it is likely that modulation of IGF-II receptor number and/or affinity may be another potential control point for the regulation of IGF-II actions.

The IGFBPs represent the third important component of the IGF-II regulatory system. It is now known that the biological actions of IGFs can be modulated by the IGFBPs and that the IGFBPs are regulated by a number of systemic and local factors. Therefore, the biological actions of IGFs depend not only on the IGFs themselves but also on the IGFBPs. In this regard, recent studies have shown that human bone cells in culture produce a number of IGFBPs (25, 29, 34, 38.5, and 41.5 kd forms), of which the 25 kd form is the most abundant.[52] The 25 kd inhibitory IGFBP now designated as IGFBP-4 has high affinity for IGF-I and II and is a potent inhibitor of IGF actions in bone cells.[53] Recent findings have shown that inhibition of TE85 cell proliferation by agents (prostaglandin E2, forskolin, isobutylmethylxanthine) which increase intracellular cAMP is accompanied by an increased IGFBP-4 production thus suggesting that IGFBP-4 may act as an important determinant of bone cell proliferation by modulating IGF activity.[40] Consistent with this interpretation, it was found that agents which increased human bone cell proliferation (IGF-I and II, human growth hormone) decreased production of IGFBP-4.[54] Furthermore, the finding that the level of IGFBP-4 in the conditioned medium of TE85 cells correlates to the level of cell proliferation[40] suggests that IGFBP-4 is another important determinant of bone cell proliferation.

Because bone cells produce IGF-II, IGFBPs and receptors for IGF-II, we propose that the actions of IGF-II are regulated by the interaction of these three variables. Since IGFs are produced by a number of different cell types and the actions of IGFs can be different in different tissue types, it is also possible that differential regulation of the above three variables could be a possible explanation for the tissue specificity of IGF-II actions. In bone, an increase in bone formation from the perspective of the three variables discussed could result from 1) increased IGF-II production, 2) increased

IGF–II receptor affinity and/or number and 3) decreased IGFBP-4 production. These mechanisms could work independently, i.e., two variables remain constant while the third changes or in combination depending upon the conditions and requirements. Since bone cells produce other growth factors and IGFBPs, it is quite likely that other factors beside IGF-II and IGFBP-4 participate in the regulation of bone cell metabolism and the coupling phenomenon. However, the evidence presented in this review suggests that the IGF-II system in bone plays a key role in bone cell regulation.

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FIGURE LEGEND

Figure 1

Model illustrating the storage and autocrine/paracrine actions of growth factors in bone. Growth factors produced by osteoblasts diffuse either into the bone matrix where the factors are stored or into the extracellular fluid where the factors can affect bone cells via autocrine/paracrine mechanisms.

Figure 2

Model illustrating the delayed paracrine action of growth factors fixed in bone by a bone derived-IGFBP (BD-IGFBP). Osteoclastic resorption of an area of bone releases the growth factor and the BD-IGFBP both of which can then act on precursor osteoblasts and mature osteoblasts to insure site-specific bone replacement.
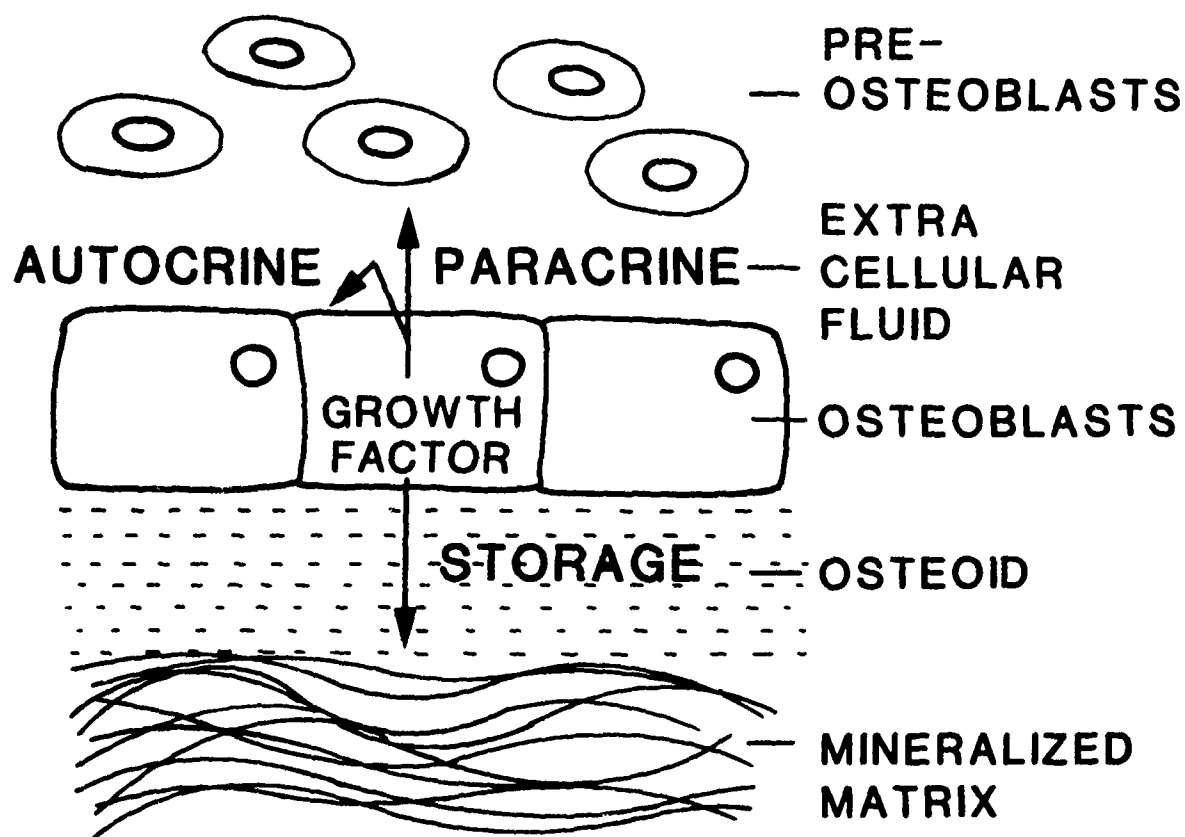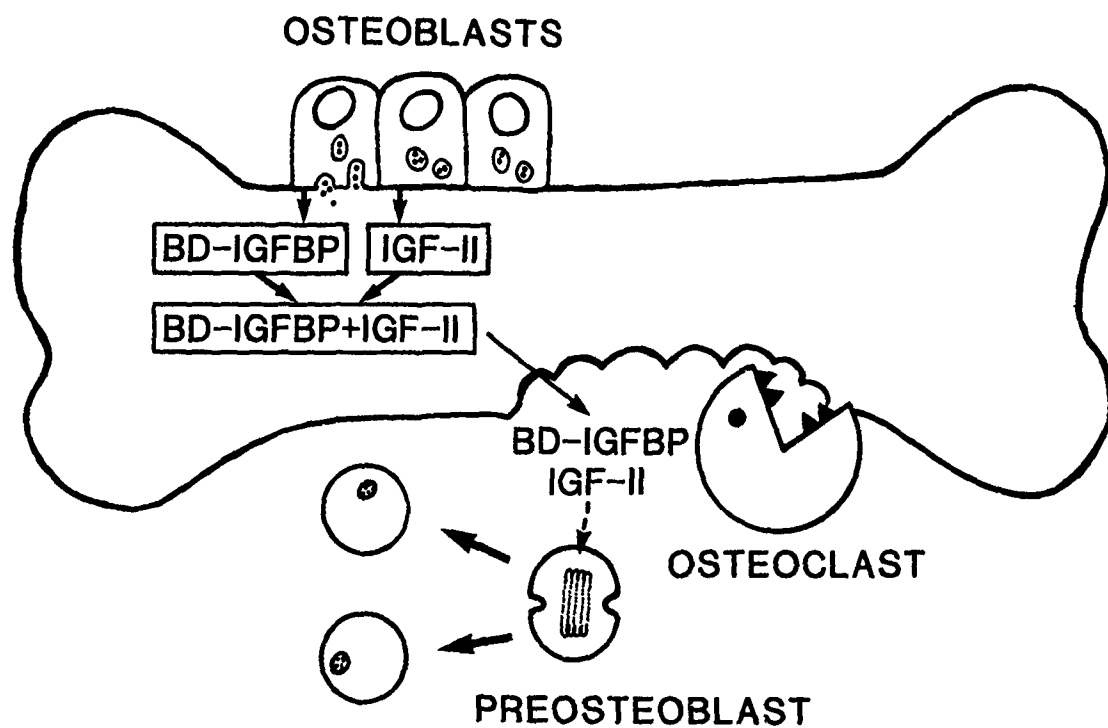
PRE-
OSTEOBLASTS

AUTOCRINE PARACRINE

EXTRA
CELLULAR
FLUID

GROWTH
FACTOR

OSTEOBLASTS

STORAGE

OSTEOID

MINERALIZED
MATRIX

Figure 1

OSTEOBLASTS

BD-IGFBP   IGF-II

BD-IGFBP+IGF-II

BD-IGFBP
IGF-II

OSTEOCLAST

PREOSTEOBLAST

Figure 2

391